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A NEW GAS CHROMATOGRAPHIC METHOD FOR THE ESTIMATION OF RESERPINE AND RESCINNAMINE*

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SUMMARY

Under the conditions described for alkaline hydrolysis of reserpine and rescinnamine in absolute and aqueous methanol, and after esterification (with diazomethane) of the resulting acid fraction, methyl 3,4,5-trimethoxybenzoate was quantitatively recovered, whereas methyl *trans*-3,4,5-trimethoxycinnamate, in normal lighting conditions, was either partly isomerized to methyl *cis*-trimethoxycinnamate or formed an adduct with a molecule of methanol, yielding methyl 3-methoxy-3-(3,4,5-trimethoxyphenyl)propionate. The structures of the products were established by synthesis, nuclear magnetic resonance studies and mass spectrometry. This investigation of the hydrolytic conditions allowed a reliable and rapid gas chromatographic determination of reserpine and/or rescinnamine in amounts down to 500 and 2000 μg, respectively, to be devised.

INTRODUCTION

There are many methods for qualitative and quantitative determination of reserpine and/or rescinnamine in the literature. Such methods can be classified, according to the technique used, into direct spectrophotometric (UV and visible), colorimetric, fluorimetric, chromatographic, potentiometric or electrophoretic procedures (see refs. 1–6 and references cited therein), and indirect hydrolytic ones, these last-named being followed by spectrophotometric determination of the free trimethoxybenzoic acid (TMBA)[7,8] and/or trimethoxycinnamic acid (TMCA)[9].

Many quantitative control determinations of reserpine and/or rescinnamine in pharmaceuticals are based on these hydrolytic methods. Such a determination, within the reported limits[1], appears to be acceptable only for reserpine; a similar determination of rescinnamine by UV spectrophotometry of the free TMCA cannot be con-

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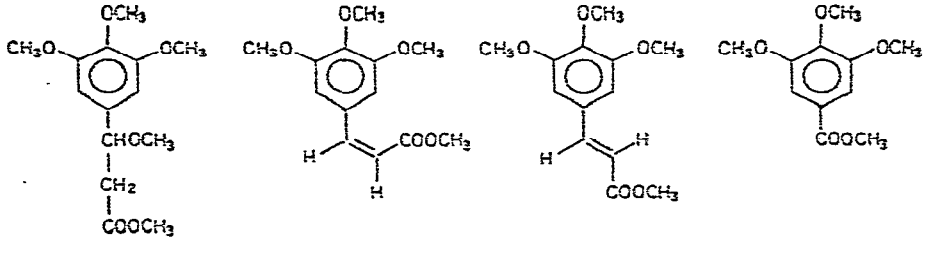
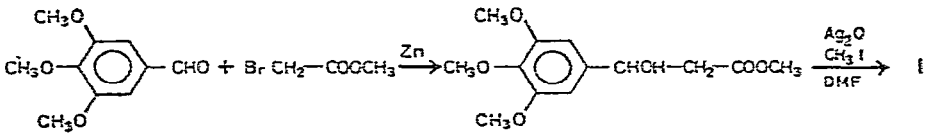
sidered as reliable. Thus, quantitative analysis of mixtures of reserpine and rescinnamine by a method involving UV absorption, making use of a two-point correction technique[9], is of no practical use. The sensitivity of TMCA to daylight and to heat is the cause of unreliability in UV determinations of rescinnamine: the involved alteration of the molecule has been attributed to *cis–trans* isomerism of TMCA[10].

Our present work was directed first at identifying the transformation products of TMCA in the mixture obtained on alkaline hydrolysis of rescinnamine, and verifying that no alteration of TMBA occurred under the same conditions. Thus, we submitted methyl 3,4,5-trimethoxybenzoate (MTMB; IV) and methyl *trans*-3,4,5-trimethoxycinnamate (*trans*-MTMC; III) to alkaline hydrolysis, and determined the resulting methyl esters of TMBA, TMCA and the derived acids by gas chromatography (GC). To our knowledge, no direct or indirect GC determination of the two alkaloids has been reported in the chemical literature.



I II III IV



On alkaline hydrolysis and esterification of the resulting mixture with diazomethane, *trans*-MTMC yielded three products identified by GC as described later, *viz.*, methyl 3-methoxy-3-(3,4,5-trimethoxyphenyl)propionate (I), *cis*-MTMC (II) and unchanged III.

These compounds were isolated by column chromatography and identified by means of their NMR and mass spectra (see Experimental).

Moreover, I was prepared by a Reformatsky condensation of 3,4,5-trimethoxybenzaldehyde with methyl bromoacetate, followed by methylation of the alcoholic hydroxyl group of the obtained methyl 3-hydroxy-3-(3,4,5-trimethoxyphenyl)propionate (see reaction scheme above).

cis-MTMC (II) was also prepared through UV irradiation of a methanolic solution of III, as described for *trans*-cinnamic acid[11,12], followed by chromatographic separation on a column of acetylcellulose.

MTMB (IV) was quantitatively recovered after alkaline hydrolysis and diazomethane esterification of the resulting acid.

The features of the UV absorption spectra of I–III are clearly different; Fig. 1 shows their molar absorption curves in methanol solution (and that of IV), and their NMR and UV spectral data are given in Table I.

It is obvious from Fig. 1 that rescinnamine determinations based on UV spectrometry of free TMCA will be unreliable; moreover, variations can occur in the relative proportions of the resulting I–III.

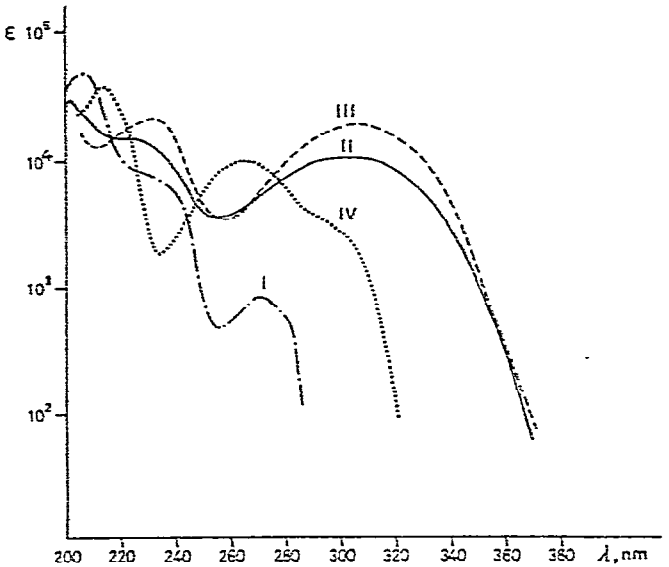


Fig. 1. UV molar absorption curves of compounds I–IV in methanol solution.

EXPERIMENTAL

Materials and apparatus

All solvents and reagents were of analytical grade and were used as received, unless otherwise stated.

MTMB and *trans*-MTMC were prepared by diazomethane esterification of TMBA and *trans*-TMCA (Fluka, Buchs, Switzerland). Pure MTMB melted at 81° (*n*-hexane); pure *trans*-MTMC melted at 98° after repeated crystallization from cyclohexane.

Reserpine and rescinnamine (Fluka) were respectively purified from 80% aqueous acetone and benzene, and complied with the requirements of the eighth editions of the Farmacopea Italiana and the Merck Index.

Column chromatography was carried out on silica gel 60, 70–230 mesh, (E. Merck, Darmstadt, G.F.R.) and on acetylcellulose (Woelm, Eschwege, G.F.R.).

The GC was performed with a Perkin-Elmer 900 instrument equipped with a flame ionization detector, a Hitachi Perkin-Elmer 159 recorder (1 mV full-scale) and an electronic digital integrator (Hewlett-Packard HP 3373B).

Mass spectra were obtained with a Perkin-Elmer 270 spectrometer operated at

TABLE I

NMR AND UV DATA FOR COMPOUNDS I–IV

Compound	*NMR chemical shift (δ), ppm**		*UV spectral parameters*	
			$\lambda_{max.}$, *nm*	ε
I	6.48 s(2)	3.63 s(3)	208**	46,300**
	4.45 q(1)	3.20 s(3)	270**	790**
	3.85 s(6)	2.53 q(2)	207***	46,800***
	3.72 s(3)		270***	910***
II	7.11 s(2)		203**	28,900**
	6.65 d(1)		223**	14,500**
	5.72 d(1)		310**	13,500**
	3.80 s(6)		202***	28,700***
	3.75 s(3)		223***	15,500***
	3.66 s(3)		302***	11,800***
III	7.42 d(1)	3.70 s(3)	233**	21,100**
	6.60 s(2)	3.66 s(3)	307**	19,200**
	6.12 d(1)		233***	21,500***
	3.80 s(6)		304***	19,400***
IV	7.10 s(2)		216**	33,200**
	3.80 s(9)		263**	10,300**
	3.73 s(3)		213***	36,500***
			265***	10,100***

* Values for solution in carbon tetrachloride: s = singlet; d = doublet; q = quadruplet. The figures in parentheses indicate the numbers of protons integrated.

** For solution in cyclohexane.

*** For solution in methanol.

70 eV (100 μA, 2 kV) with a helium flux, and UV spectra (for cyclohexane and methanol solutions) with a Cary 17 spectrophotometer.

NMR spectra were recorded (on a Varian T60 spectrometer) for solutions in carbon tetrachloride, and chemical shifts were reported relative to tetramethylsilane as internal standard.

Melting points reported are uncorrected.

Isolation and identification of compounds produced from trans-MTMC in the hydrolytic conditions used for rescinnamine

Pure *trans*-MTMC (1.2 g) was heated under reflux for 3 h in 50 ml of absolute methanol containing 140 mg of sodium hydroxide. After cooling and neutralization with dilute hydrochloric acid, the methanolic solution was treated with an excess of ethereal diazomethane, allowed to react for a few minutes at room temperature, then evaporated to dryness. The residue was dissolved in chloroform and chromatographed on a silica gel column (35 cm × 3 cm I.D.), with chloroform as eluent; fractions of 50 ml were collected. Fractions 3–8 contained mainly *cis*- and *trans*-MTMC (II and III), whereas methyl 3-methoxy-3-(3,4,5-trimethoxyphenyl)propionate (I), the main product, was eluted in fractions 9–16.

After removal of the solvent, the residue from fractions 3–8 (251 mg) was chromatographed on an acetylcellulose column (90 cm × 3.2 cm I.D.), with *n*-hexane

as eluent and collection of 30-ml fractions; *cis*-MTMC (II), containing about 2% of III, was first eluted (146 mg). Pure II (m.p. 47–49° as reported[13] and confirmed by GC) was obtained through repeated column chromatography on acetylcellulose: its mass spectrum was identical with that of III (M^+ at *m/e* 252).

The residue of fractions 9–16 (142 mg) was again chromatographed on a silica gel column (37 cm × 1.5 cm I.D.), with chloroform as eluent and collecting 15-ml fractions: pure I (112 mg; m.p. 67–69° after crystallization from cyclohexane) was obtained. Its mass spectrum showed the molecular-ion peak M^+ at *m/e* 284.

Analysis for $C_{14}H_{20}O_6$. Found: H = 6.98%; C = 59.03%; OCH_3 = 54.27%. Calculated: H = 7.09%; C = 59.14%; OCH_3 = 54.50%.

Methyl 3-hydroxy-3-(3,4,5-trimethoxyphenyl)propionate

This compound was prepared as described for a similar Reformatsky condensation[14] from 59 g (0.3 mole) of 3,4,5-trimethoxybenzaldehyde, 38 g (0.25 mole) of methyl bromoacetate and 20 g (0.3 g-atom) of activated zinc dust. The yield was 19.3 g (24%), and the product melted at 94° after crystallization from carbon tetrachloride.

Analysis for $C_{13}H_{18}O_6$. Found: H = 6.68%; C = 57.84%. Calculated: H = 6.71%; C = 57.77%.

NMR data ($CDCl_3$). Chemical shift (δ), ppm: s(2) 6.60; t(1) 50.3; s(9) 3.82; s(3) 3.70; s(1) 3.44; d(2) 2.70.

Methyl 3-methoxy-3-(3,4,5-trimethoxyphenyl)propionate (I)

To a solution of 9.2 g (0.034 mole) of methyl 3-hydroxy-(3,4,5-trimethoxyphenyl)propionate and 6.9 ml (0.11 mole) of iodomethane in 70 ml of freshly distilled dimethylformamide in a tightly sealed actinic-glass vessel were slowly added 8.5 g of washed and dried silver oxide (0.037 mole) at room temperature; there was no increase in temperature during the addition. The suspension was stirred for 24 h in the dark at room temperature, then centrifuged, and the resulting precipitate was thrice rinsed with 3 volumes of chloroform; the rinsings were combined with the previously separated dimethylformamide solution. From the resulting clear and colourless solution the chloroform-insoluble complex $2AgI \cdot NH(CH_3)_2$ (ref. 15) separated (the separation was completed in a refrigerator). The cold solution was filtered, and solvent was evaporated under reduced pressure from the filtrate. The resulting viscous yellow residue (which crystallized on standing) was found by GC to be a mixture of I and III.

These two compounds were separated by fractional crystallization from cyclohexane (I was the more soluble) and purified by chromatography on a silica gel column (80 cm × 3.3 cm I.D.), with dichloromethane as eluent. I was eluted first: it crystallized from cyclohexane in colourless needle-shaped druses (m.p. 69–70°). The yield was 4.3 g (46%). By means of GC, NMR and mass spectrometry, the product was shown to be identical with the compound obtained through alkaline hydrolysis of *trans*-MTMC (see Table I).

UV irradiation of trans-MTMC

A solution of 2.12 g of *trans*-MTMC in 150 ml of methanol was irradiated for 1 h by an immersed UV source (Quartzlampen, Hanau, G.F.R.), cooling externally with ice and water: the internal temperature was maintained at 28–30°.

Tests by GC revealed that the *cis*-to-*trans* ratio reached its maximum value after 1 h; therefore, longer irradiation would lead only to an increased formation of resinous polymerization products.

After evaporation of the methanol, the residue obtained was chromatographed on an acetylcellulose column in subdued daylight, eluting with *n*-hexane as previously described. This column chromatography was repeated, and 420 mg of pure *cis*-MTMC (m.p. 47–49°) were thus obtained.

PROCEDURE

Preliminary observations

The methods for alkaline hydrolysis are essentially those reported for reserpine and rescinnamine[7–9,16], but under two different sets of conditions, *viz.*, with sodium hydroxide in absolute methanol, and with sodium hydroxide in aqueous 80% methanol in constant-temperature conditions. In both instances, the base concentration was the same (0.03 *M*), and was chosen in order to minimize formation of I without diminishing its hydrolytic power.

The diazomethane methylation of the acids obtained by hydrolysis of rescinnamine, when not carried out under controlled conditions (freshly prepared diazomethane, and esterification at 0° for 25 min) also led to the formation of by-products (not revealed by GC), possibly resulting from the addition of diazomethane to α,β-unsaturated esters[17,18]. The yields of II and III were thus diminished.

Equal amounts of *trans*-MTMC were hydrolyzed under the same conditions as used for rescinnamine with methanolic sodium hydroxide: when the resulting products were methylated with freshly prepared diazomethane at 0° for 25 min, the total recoveries of I, II and III ranged from 92 to 100%. When methylation was carried out at room temperature for the same time, the over-all recoveries of I, II and III ranged from 60 to 96% (depending on the room temperature).

Gas chromatographic analysis of reserpine and/or rescinnamine

The sample of purified reserpine and/or rescinnamine (in the amounts shown in Table II) was heated under reflux for 90 min in 5 ml of the chosen hydrolysis reagent, the temperature of the water-bath being kept constant at 70–75°. After cooling, the pH of the solution was adjusted to 4–5 with 0.1 *N* hydrochloric acid (about 2.5 ml), then the mixture was diluted with 10 ml of distilled water, transferred to a 100-ml separating-funnel and extracted with three 15-ml portions of peroxide-free diethyl ether; the combined ether extracts were collected in a flask, and the solvent was evaporated. The water always present in the residue was removed by addition of small volumes of benzene–absolute ethanol mixtures and distillation. The residue was then dissolved in a few millilitres of peroxide-free diethyl ether and cooled at 0° with ice–water; 1 ml of freshly prepared ethereal diazomethane solution[19] was then added, and the mixture was allowed to react for 25 min at 0°.

The excess of diazomethane and the solvent were removed under reduced pressure from the cold solution, and 1 ml of a dichloromethane solution of the internal standard (methyl stearate; 1 mg/ml) was added to the residue. The resulting methyl esters (I–IV) and the internal standard were dissolved in carbon disulphide and quantitatively transferred to a 5-ml volumetric flask, and this solution, after being

diluted to volume with carbon disulphide, was submitted to GC under the conditions described below.

A typical chromatogram for the analysis of a mixture of 500 μg of reserpine and 2000 μg of rescinnamine is shown in Fig. 2. The retention times of compounds I–IV, relative to that of the internal standard are as follows: I, 0.30; II, 0.37; III, 0.60; IV, 0.19.

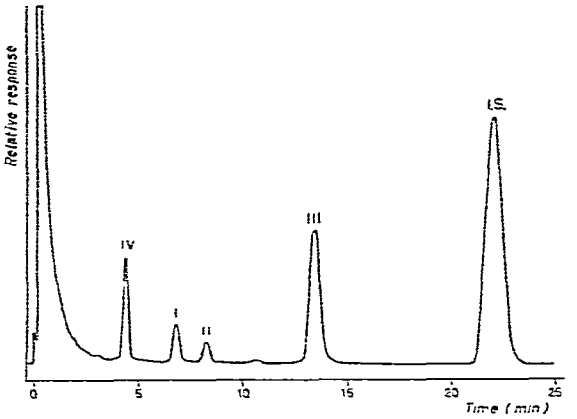


Fig. 2. Gas chromatogram of compounds I–IV resulting from the alkaline hydrolysis of a reserpine–rescinnamine mixture (500 μg + 2000 μg).

Conditions for GC

Glass column: 240 cm × 0.2 cm I.D.

Stationary phase: 15% of Apiezon L (purified by column chromatography on alumina) on Chromosorb W (AW; 80–100 mesh).

Sample volume: 5 μl; injected with a Glenco 19909-10 micro-syringe.

Carrier gas: nitrogen (puriss.) at a flow-rate of 30 ml/min.

Auxiliary gases: hydrogen (30 ml/min); dry air (300 ml/min).

Temperatures: column, 230°; injector, 260°: detector, 200°.

Recorder-chart speed: 10 mm/min.

Determination of esters I–IV was accomplished by means of calibration curves of the relative response factors of the pure compounds established from the ratios between their peak areas and the peak area of the internal standard.

As the aim of this work was to achieve rapid determination of total amounts of the two alkaloids in the range 500–2000 μg, the calibration curves were drawn to cover the concentration interval 25–150 μg per 5 ml for I and II, and 100–600 μg per 5 ml for III and IV. Over these intervals, the relationship between quantities and GC responses was rectilinear.

RESULTS AND DISCUSSION

The results obtained are reported in Table II: they indicate that sodium hydroxide in absolute methanol is the best hydrolytic reagent.

As hydrolysis of reserpine always leads only to TMBA, whereas, in the same circumstances, rescinnamine yields *trans*-TMCA, *cis*-TMCA and 3-methoxy-3-(3,4,5-trimethoxyphenyl)propionic acid, the sensitivity of the method for rescinnamine is

TABLE II

PERCENTAGE RECOVERIES OF COMPOUNDS I–IV AFTER HYDROLYSIS OF RESERPINE–RESCINNAMINE MIXTURES

Hydrolysis reagent	*Amount of rescinnamine (μg) mixed with 500 μg of reserpine*	*I*	*II*	*III*	*I + II + III*	*IV*
Sodium hydroxide–methanol	500	11.1 ± 2.6	2.7 ± 2.5	67.4 ± 3.4	81.0 ± 5.0*	92.4 ± 4.7*
	1000	13.1 ± 1.8	4.3 ± 2.4	69.1 ± 5.5	86.6 ± 6.3**	94.2 ± 3.6**
	2000	12.7 ± 1.7	9.7 ± 1.6	74.6 ± 5.1	97.0 ± 5.6***	96.4 ± 4.2***
Sodium hydroxide–80% aq. methanol	2000	5.1 ± 2.5	9.2 ± 1.5	73.1 ± 3.8	87.3 ± 4.8§	92.2 ± 3.7§

* Calculated on 6 determinations.
** Calculated on 8 determinations.
*** Calculated on 12 determinations.
§ Calculated on 4 determinations.

affected by the calculation errors for three peak areas. Thus, although the method is reliable for reserpine determinations down to 500 μg, for rescinnamine it is satisfactory only for quantities down to 2000 μg. However, consistent recoveries of I–III were obtained from minor quantities of rescinnamine (see Table II).

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