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COUPLING OF CHROMATOGRAPHIC TECHNIQUES WITH MICRO-INFRARED SPECTROMETRY FOR THE DETERMINATION OF PHENOTHIAZINE AND RELATED DRUGS

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SUMMARY

Several experiments that were carried out to isolate phenothiazine drugs from TLC plates for micro-infrared spectroscopic examination were not successful. A system for combining preparative gas–liquid chromatography with micro-infrared spectroscopy was evaluated. A splitter was installed that enabled us to trap each chromatographed phenothiazine or related compound on the microgram scale. All the substances obtained were highly pure, which made micro-infrared spectroscopy an easy task.

INTRODUCTION

Infrared (IR) spectroscopy, being essentially an additive method of high specificity, is most promising if it is applied after the separation of compounds by chromatographic techniques.

MORGAN[1] developed a method in which paper chromatography was combined with IR spectroscopy for identifying organic bases such as morphine, and McCOY AND FIEBIG[2] described a new TLC–IR coupled technique.

Many systems for the isolation of substances after GLC separation in the gram[3–8], milligram[9–14] and microgram ranges[15,16], have been described. Microgram amounts of lipids[16–19], steroids[20], pesticides[21] and narcotics[22] have been determined by combined GLC–IR techniques.

It was our purpose to develop TLC and GLC–IR combined techniques for the determination of phenothiazine drugs and related derivatives. A simple preparative GLC trapping system permitting the subsequent spectrometric examination of the compounds isolated was devised.

EXPERIMENTAL

Thin-layer chromatography

Three series of 250-μm thin-layer plates were prepared: (1) equal parts of Silica Gel HF_{254} and Cellulose MN-300, (2) Silica Gel HF_{254} and (3) Silica Gel H. All

plates were eluted with the solvent system chloroform–acetone–ammonia, 25 % (50:50:1), and dried in air before use. Amounts of 200 μg of promazine hydrochloride (20 μl of a 1 % solution in ethanol) were spotted in a 2-cm line on 3 cm of the lower edge of each plate; a supplementary reference spot of 10 μg of promazine hydrochloride was applied only on the Silica Gel H plates.

After development over a 13-cm distance in the above system, the plates were dried for 15 min in a vacuum oven at 50° and 4 mm Hg pressure. On TLC plates with a fluorescent indicator, the promazine spots were located by direct inspection under 254-nm UV light. Chromatograms with Silica Gel H as adsorbent were covered with a glass plate, except for the track with the 10-μg reference spot. This spot was made visible by spraying with a ferric reagent[23], which was prepared by dissolving 500 mg of $Fe(NO_3)_3$ in 80 ml of conc. H_2SO_4 and diluting to 1000 ml with distilled water. The promazine spots, together with an equal amount of blank adsorbent, were scraped off and eluted as described below.

Thin-layer chromatographic elution procedures

Direct elution. The adsorbent fractions isolated were separately poured on to a G_2 filter and eluted five times with 1 ml of freshly distilled methanol.

Two-phase extraction. Aliquots of the adsorbent fractions were separately transferred into 15-ml centrifuge tubes, fitted with glass stoppers, and extracted five times with 5 ml of 0.1 *N* HCl. In a first series of experiments, the combined acidic extracts were extracted twice with 30 ml of freshly distilled chloroform. In a second series of experiments, the acidic extracts were made alkaline with 1 ml of 10 *N* NaOH and extracted twice with 30 ml of peroxide-free diethyl ether (freshly distilled over reagent-grade hydroquinone). The extracts were dried over Na_2SO_4, filtered and concentrated with the aid of a slow stream of nitrogen on a water-bath at 60° to a final volume of 1 ml.

Continuous extraction. Aliquots of the adsorbent fractions were continuously extracted for 1 h with 1 ml of methanol, 1 ml of ethanol or 1 ml of chloroform using the apparatus described by HAYDEN *et al.*[24].

Gas–liquid chromatography

Gas–liquid chromatography was performed with a Hewlett-Packard research gas chromatograph, Model 5750, equipped with a dual flame ionization detector (FID) and a catharometer detector. Two packed columns of different polarity were installed for simultaneous single-column operation.

The polar column was prepared with 1 % FFAP (a polyester, the reaction product of Carbowax 20 M and *m*-dinitroterephthalic acid) by coating the liquid phase on Diatoport S (acid and silane treated), 80–100 mesh (Hewlett-Packard). One of the following columns was used as an apolar system: 3.8 % SE-30 or 5 % OV-1 (a methylsilicone polymer) also coated on Diatoport S (acid and silane treated), 80–100 mesh. The operating conditions were: glass spiral columns, 1.80 m long and 4 mm I.D.; carrier gas, nitrogen, 70 ml min^{-1} (bubble flow meter); oven temperature as indicated later in Table II; injector and detector temperatures about 10° higher than the oven temperature; hydrogen, 7.0 ml min^{-1}; air, 80 ml min^{-1}.

Volumes varying from 1 to 50 μl containing 10 to 200 μg of active substance were injected and chromatographed for micro-preparative purposes.

Trapping system for GLC effluents on the micro-scale

A Hewlett-Packard splitting system was installed at the exit of each column, and split the effluent gas so that ten parts passed to the FID and a hundred parts entered the catharometer block. This splitting ratio was obtained by passing the effluent through two parallel-mounted capillaries of length 101.6 cm (FID) and 10.16 cm (catharometer) (40 in. and 4 in., respectively). The catharometer detector was heated at the same temperature as the oven and used only as a transfer zone (bridge current switched off).

As a collection device, a Pyrex capillary of 12.5 cm length and 1.5 mm I.D., fixed with two PTFE O-rings in a brass tube of 10.5 cm length, 7 mm O.D. and 4 mm I.D., and provided with a 0.42-cm (0.125-in.) swagelock nut, was used. For each collection, a brass tube was first loaded with a carefully cleaned capillary (stored overnight in chromic acid, rinsed six times with distilled water, and dried at 120° in a drying oven), and the assembly was attached to the exit of the catharometer block approximately 1 min before the desired compound was eluted.

Infrared spectrometry

The 1-ml elutions from the TLC plates were evaporated under vacuum at 60° and the residues transferred with three 50-μl volumes of ethanol on to 50 mg of KBr in an agate mortar. When the solvent had evaporated off, a pellet was prepared by means of a punched disk (2 × 10-mm hole, 13 mm diameter). The compound collected after GLC was rinsed from its capillary with two 10-μl volumes of chloroform, ethanol or ethyl acetate. These solutions were poured on to 27 mg or 6–8 mg of KBr in an agate mortar, and the solvent was allowed to evaporate. A micropellet was prepared by means of a punched disk (2 × 10-mm or 1 × 4-mm hole, 13 mm diameter). IR spectra were recorded from 625 to 4000 cm^{-1} with a Perkin-Elmer 257 spectrophotometer, equipped with an RIIC C-41 microbeam condenser.

Ultraviolet spectrometry

For determining the GLC trapping efficiency, compounds were quantitatively rinsed from their capillaries with 3.0 ml of 0.1 *N* HCl and UV spectra were automatically recorded with a double-beam Unicam SP800 spectrophotometer in the wavelength range 450–190 nm.

Reference substances

Free bases. These were prepared as follows: 100.0 mg of the salt form was dissolved in 20 ml of distilled water, acidified with 1 ml of 0.1 *N* HCl and quantitatively transferred to a 100-ml separating funnel. After saturation with nitrogen, 1 ml of 10 *N* NaOH was added and the solution extracted twice with 50 ml of peroxide-free ether. The separated and combined ethereal phases were dried over Na_2SO_4, filtered, evaporated by means of a nitrogen stream to a final volume of about 1 ml and diluted to a total volume of 10.0 ml. Solutions were stored at −20°.

Salts. Salts were used in their commercially available forms. Solutions were prepared by dissolving 100.0 mg amounts in 10.0 ml of ethanol.

Sulphoxides. Sulphoxides were prepared as follows: 100.0 mg of each compound were transferred to a 10.0-ml calibrated cylinder, dissolved in 1 ml of 15% H_2O_2 and 0.2 ml of acetic acid was added; after a reaction time of 30 min in a water-bath at 60°, the reaction mixture was diluted to 10.0 ml.

Acetylated derivatives. These were formed as follows: 10.0 mg of each compound were placed in a 15-ml conical siliconized tube and 0.2 ml of pyridine (refluxed and distilled over KOH) and 0.2 ml of acetic anhydride (refluxed and distilled over calcium carbide) were added; after a reaction time of 1 h in a P_2O_5 desiccator, the reaction mixtures were evaporated under a slow stream of nitrogen and the residues obtained were dissolved in 10.0 ml of ethyl acetate (0.1 % solutions).

RESULTS AND DISCUSSION

IR spectra of promazine, isolated by direct elution or two-phase extraction from TLC plates, were compared with blank eluents and did not yield satisfactory results. All spectra gave the same high background absorption pattern that interfered completely with the true promazine pattern. The presence of higher amounts of impurities originating from the adsorbents or solvents were a dominant feature. Relatively large volumes (5 ml of methanol or 2 × 30 ml of chloroform) were used in the procedure and resulted in the concentration of impurities. Repetition of the above experiment by applying 1.2 mg of promazine hydrochloride on to the TLC plate and preparing a pellet with 300 mg of KBr gave an IR spectrum that was comparable with the reference pattern. Under these conditions, impurities became six times triturated while the promazine concentration remained constant. Examination of the IR spectra from promazine and promethazine isolated from TLC plates by continuous extraction did not give better results. Again, blank experiments revealed that contamination was too high. Although a much smaller extraction volume (1 ml) was used in these experiments, the extraction was more drastic, which afforded the same overall contamination effect.

The failure of the experiments described above probably originated from the use of highly polar eluents such as methanol and chloroform. On this basis they should not be considered to be in contradiction to more successful TLC–IR combinations[24].

For GLC trapping, we preferred to use a Hewlett-Packard splitter. According to Poiseuille's law, the column effluent split over two parallel-mounted capillary restrictions of identical diameter and different lengths affords a ratio $D_{\text{flame}}/D_{\text{cath.}}$ that is inversely proportional to the respective lengths, $l_{\text{flame}}/l_{\text{cath.}}$. When $l_{\text{cath.}} = 4$ in. and $l_{\text{flame}} = 40$ in., 100 parts of effluent pass through the catharometer detector while 10 parts enter the FID.

The brass tube used for holding the Pyrex capillary during collection served as a temperature gradient which avoided high losses of trapped compound through aerosol formation. The simultaneous recording of the chromatogram allowed us to follow the chromatographic process and indicated the moment at which the collection device could be unscrewed from the chromatograph (Fig. 1). Experimentally, several sources of contamination were successfully eliminated. Sufficient conditioning of the GLC column made column bleeding a minor problem. Control experiments were carried out by collecting for 20 min column effluent from 1 % FFAP on Diatoport S, 80–100 mesh, at 245° and 5 % OV-1 on Diatoport S, 80–100 mesh, at 260°. No interfering IR absorptions were observed under these forced operating conditions. Chromic acid was used for cleaning the Pyrex capillaries and all manipulations were performed by means of a pair of clean tweezers (contact with the fingers has to be avoided). As

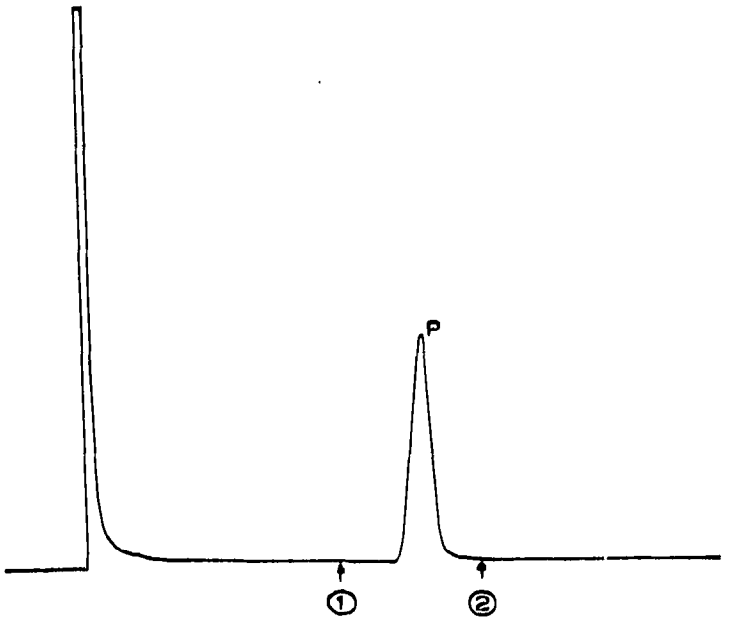

Fig. 1. Preparative GLC chromatogram of perazine. Stationary phase, 1% FFAP; oven temperature, 240°; amount injected, 49 μg. 1 = Point where collection device was mounted; 2 = point where collection device was uncoupled.

TABLE I

RESULTS OF TRAPPING EFFICIENCY USING A 3.8% SE-30 COLUMN AT 220°

Compound	*Amount injected on to the column (μg)*	*Recovery (%)*
Amitriptyline	37	62
Chlorpromazine	37	51
Levomepromazine	36	53
Promazine	37	53
Promethazine	34	61

solvents for effecting transfers, two 10-μl volumes of chloroform, ethanol or ethyl acetate gave good solubilization of the compounds examined without yielding too much contamination. Ethyl acetate seemed to be the most useful solvent for acetylated derivatives.

The trapping efficiency of our micro-preparative GLC system was determined by quantitative UV spectrophotometry. The results are summarized in Table I.

The overall yield for this class of compounds varied from 51 to 62%. Situated in the low microgram range, namely 34–37 μg, this yield is satisfactory for qualitative micro-IR examination.

The results of successful GLC–IR experiments using 50–80 μg amounts of sample are given in Table II.

IR spectra of all the compounds mentioned in Table II were identical with reference spectra. An example is given for perazine in Fig. 2.

TABLE II

PHENOTHIAZINE AND RELATED DERIVATIVES EXAMINED BY THE GLC–IR SYSTEM

Compound	*Column system*	*Oven temperature (°C)*
Free bases		
Dibenzepine	1% FFAP	240
Imipramine	5% OV-1	190
Levomepromazine	1% FFAP	230
	5% OV-1	185
Nortriptyline	5% OV-1	185
Perazine	1% FFAP	240
Prothipendyl	1% FFAP	230
Thioproperazine	5% OV-1	260
Trifluoperazine	1% FFAP	230
Salts		
Perazine dimalonate	5% OV-1	240
Prothipendyl hydrochloride	1% FFAP	230
Thioproperazine bismethane-sulphonate	5% OV-1	260
Trifluoperazine dihydrochloride	1% FFAP	230
Sulphoxides		
Levomepromazine sulphoxide	5% OV-1	230
Perazine sulphoxide	5% OV-1	240
Prothipendyl sulphoxide	5% OV-1	230
Trifluoperazine sulphoxide	5% OV-1	230
Acetylated derivatives		
(1) *Acetyl esters*		
Dixyrazine acetyl ester	5% OV-1	260
2-Hydroxyiminodibenzylacetyl ester	1% FFAP	240
2-Hydroxyimipramine acetyl ester	1% FFAP	240
Propericiazine acetyl ester	5% OV-1	260
(2) *Acetamides*		
Bidesmethylimipramineacetamide	1% FFAP	240
Desmethylimipramineacetamide	1% FFAP	240
Dibenzepine-II-acetamide[a]	1% FFAP	240
Dibenzepine-IV-acetamide[b]	1% FFAP	240
Dibenzepine-V-acetamide[c]	1% FFAP	240
Dibenzepine-VI-acetamide[d]	1% FFAP	240
Nortriptylineacetamide	1% FFAP	230
	5% OV-1	230
Desmethylnortriptylineacetamide	1% FFAP	230
	5% OV-1	230

[a] Dibenzepine-II = 5-methyl-10-β-methylaminoethyl-10,11-dihydro-11-oxo-5H-dibenzo[*b*,*e*]-[1,4]diazepine.

[b] Dibenzepine-IV = 10-β-methylaminoethyl-10,11-dihydro-11-oxo-5H-dibenzo[*b*,*e*][1,4]diazepine.

[c] Dibenzepine-V = 5-methyl-10-β-aminoethyl-10,11-dihydro-11-oxo-5H-dibenzo[*b*,*e*][1,4]diazepine.

[d] Dibenzepine-VI = 10-β-aminoethyl-10,11-dihydro-11-oxo-5H-dibenzo[*b*,*e*][1,4]diazepine.

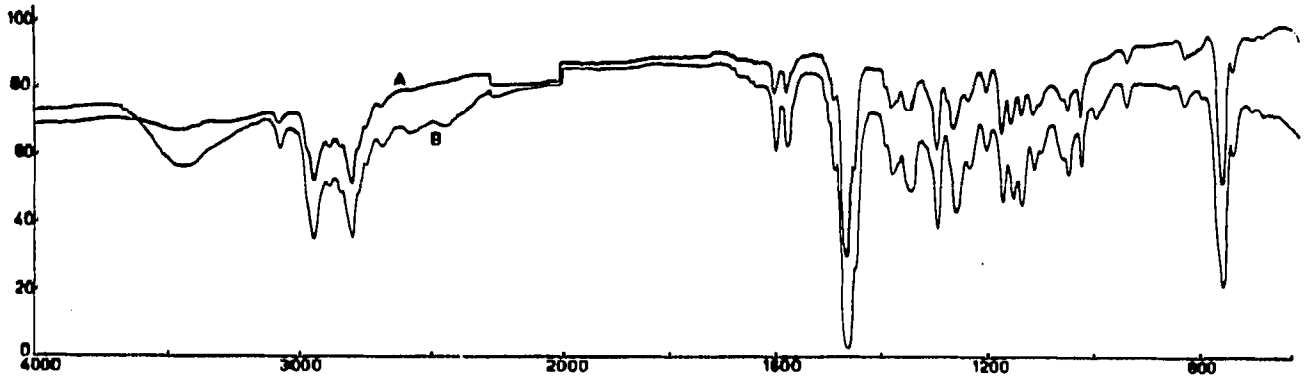

Fig. 2 (A) Micro-IR spectrum of perazine after preparative GLC. Stationary phase, 1% FFAP; oven temperature, 240°; amount injected, 49 μg. (B) Micro-IR reference spectrum of 50 μg of perazine.

The procedure enabled us to obtain particular structural information. Questions such as whether a peak corresponds to a postulated structure or whether some transformation occurred during chromatography, whether a chromatographic peak is single or composite, etc., can be answered in a simple and definite manner. Furthermore, it is evident how valuable multiple or consecutive trapping is, especially in relation to biological problems. For structure elucidation of drug metabolites isolated from biological materials, we now use routinely the described technique in our laboratories. Off-line trapping still seems to be very attractive because several complementary spectroscopic methods, *e.g.*, IR, UV and mass spectrometry, can easily be performed.

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