Journal of Chromatography, 217 (1981) 247–253
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,890

THERAPEUTIC DRUG ASSAYS WITH GAS–LIQUID CHROMATOGRAPHY AND OPTICAL DETECTION

ARTHUR KARMEN*, THOMAS PRITCHETT and STANLEY LAM
Albert Einstein College of Medicine of Yeshiva University, 1300 Morris Park Avenue, Bronx, NY 10461 (U.S.A.)

SUMMARY

Clofibric acid (*p*-chlorophenoxyisobutyric acid), the major metabolite of Clofibrate, a drug used in the treatment of hyperlipemia, was assayed in blood serum using an ultraviolet absorbance monitor as a gas–liquid chromatographic detector. As in other gas–liquid chromatographic assays for this compound, an internal standard, *p*-chlorophenoxyacetic acid, was added, and the serum was acidified and extracted with organic solvent. The solvent was then evaporated and the acids converted into their methyl esters for analysis. The organic compounds in the effluent were scrubbed into a stream of 2-propanol, at a flow-rate of 0.5 ml/min. This was then "debubbled" and a portion drawn through the 20-μl UV detector flow cell. With small-volume scrubber and associated components, peak-broadening was minimal. Because of their moderately high extinction coefficients at 280 nm, the Clofibrate and the internal standard were detected in the submicrogram range without interference from long-chain fatty acid esters, which have similar retention times on the column used.

INTRODUCTION

Several procedures have been described for applying optical detection methods in liquids to gas chromatography[1–4]. In our recent work[3,4], organic molecules were scrubbed continuously from the column effluent into a liquid stream, following which the gas and liquid phases were separated and the liquid phase delivered to the flow cell of a fluorometer or ultraviolet absorbance monitor of the kinds now used in high-performance liquid chromatography. With low-volume liquid flow-rates, the concentrations of the analytes in the scrubbing solvent were kept high, thus yielding high sensitivity. Through careful attention to the details of design of the narrow-bore scrubber, "debubbler", and detector, compounds were detected with minimal peak-broadening and loss of resolution.

We noted that optical detectors offered absolute sensitivity for many organic compounds that was comparable with that of most of the more usual gas–liquid chromatography (GLC) detectors and much greater selective sensitivity or specificity: *i.e.* the ability to detect compounds emerging from the column incompletely resolved

0021-9673/81/0000–0000/$02.50

from other kinds of compounds in the sample. For example, by scrubbing into *o*-phthalaldehyde reagent, followed by on-line recording of fluorescence, we detected sub-nanogram amounts of primary amines without interference from much larger amounts of hydrocarbons, esters and a variety of other, non-fluorescent compounds that eluted simultaneously. With UV absorbance detectors, we detected aromatic compounds in the presence of much larger amounts of aliphatics. One of the more important potential applications of specific GLC detectors in biology is in "trace" analyses, such as for hormones or drugs and their metabolites in body fluids. In the work described here we studied the analysis of Clofibrate, a drug used in the treatment of hyperlipemia, in serum using GLC with a UV detector. Several methods for assaying this drug by GLC with more conventional detectors had been described previously. Our primary objective in this work was to determine the feasibility and explore the potential advantages of using these more specific detectors.

Clofibrate is the ethyl ester of *p*-chlorophenoxyisobutyric acid (CPIB). Following its oral administration, the ester is hydrolyzed, presumably in the gastrointestinal tract, and the salt of the acid circulates in the blood serum. Several GLC methods have been described for assaying this salt[5–8]. They all involve acidifying the serum and extracting with an organic solvent, evaporating the solvent, preparing methyl esters, and analyzing them on GLC columns containing silicone liquid phases such as OV-17 or polyesters such as poly(ethylene glycol adipate) (EGA). Both the drug and its free acid are quite volatile; quantitative recovery after extraction and evaporation of the extract is somewhat difficult. Addition of known amounts of chlorophenoxyacetate and chlorophenoxyproprionate to the serum, before extracting it, as internal or recovery standards, is therefore helpful. There is an additional difficulty in that other compounds in serum elute from the column at the same time as either the methyl ester of CPIB or that of the internal standards. On columns of EGA or similar polyesters, the drug metabolites have retention times in the mid-range of the long-chain fatty acid esters present in much higher concentration in the same organic extracts of serum. On less polar silicone columns, the drug derivatives elute much earlier than the fatty acid esters but the eventual elution of the fatty acid esters confuses subsequent analyses unless the temperature is raised at the end of each analysis to "clear the column". Although selective esterification of free acids with diazomethane offers a partial solution, detection with a scheme that ignores the fatty acid esters would offer distinct advantages.

EXPERIMENTAL

Materials

Clofibric acid [2-(*p*-chlorophenoxy)-2-methyl-propanoic acid], 2-(*p*-chlorophenoxy)propanoic acid and *p*-chlorophenoxyacetic acid (CPA) were purchased from Aldrich, Metuchen, NJ, U.S.A. The methyl esters were prepared by treating the acids with 2% H_2SO_4 in methanol, adding water and extracting into light petroleum.

Equipment

The gas chromatograph was a Shimadzu Model GC-3BF. Immediately on leaving the column, the gas stream, flowing at *ca.* 30 ml/min, was split by passing it

through a stainless-steel tee tube constructed of unequal lengths of 0.25 mm I.D. stainless-steel capillary tubing, with 1/8 going to the hydrogen flame ionization detector (FID) and the rest to the gas–liquid interface, consisting of a Swagelok 1/16-in. union tee packed in the oven wall (Fig. 1). The scrubbing solvent, 2-propanol, was delivered to the tee at *ca.* 0.7 ml/min by a variable-speed infusion/withdrawal syringe pump (Harvard Apparatus Co., Model 600-2-200V) through 0.25 mm I.D. stainless-steel tubing. The output tubing of the tee, 0.75 mm I.D., containing the gas–liquid stream, was connected to the scrubbing/condensing coil, a Technicon 4 turn, 3.5-cm diameter mixing coil PN 157-0473-01, 1 mm I.D., wrapped with heat-exchanger tubing. The stream then passed to the debubbler (see also Fig. 2) from which the liquid is pulled through the UV detector flow cell through 30 cm of 0.4 mm I.D. PTFE tubing by a Buchler variable-speed Polystaltic® pump. To damp irregularities of flow the pump was connected to the flow cell through a series of restrictor tubings (3 cm × 0.4 mm I.D., PTFE). The detector was an Altex Model 153 analytical detector equipped with a 280-nm filter set and a 20-μl flow cell. The signals from both the FID and the UV detector were recorded on a linear dual-pen recorder. The columns used were: (1) coiled stainless steel, 200 cm × 1.75 mm I.D., packed with 3% OV-17 on Chromosorb WHP, 80–100 mesh; and (2) coiled nickel column, 122 cm × 2.1 mm I.D., packed with 10% EGA on Chromosorb WHP, 80–100 mesh.

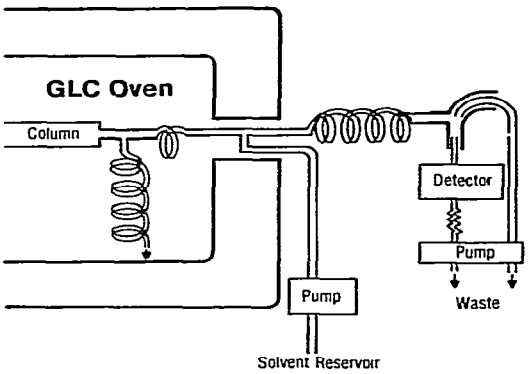

Fig. 1. Schematic diagram of the UV detection system for GLC. A stream splitter is shown in the column oven. The gas–liquid interface is shown placed in the insulation of the oven wall. The same peristaltic pump was used to draw the fluid through the detector and to draw excess fluid from the debubbler.

Preparation of samples

A stock solution of the sodium salts of CPA and CPIB was prepared to contain 20.0 μg/μl each. This solution was diluted four-fold in water. Serum samples containing 50 μg/ml were prepared by adding 5 μl of the second stock solution to 500 μl of serum. Each 500 μl sample was acidified with 500 μl of 1 *N* H_2SO_4 and extracted twice with 4% (v/v) isopropanol in benzene. The organic layers were combined and evaporated to dryness in 5-ml reaction vials. A 1-ml volume of the reaction mixture (5% H_2SO_4, 2.5% isooctane in methanol) was added to each vial. The vials were then sealed and heated at 70°C overnight, following which the mixtures were cooled to room temperature, diluted with 1 ml of distilled water, and extracted twice with 1.5 ml of light petroleum (b.p. 30–60°C). The organic layers were combined and evaporated

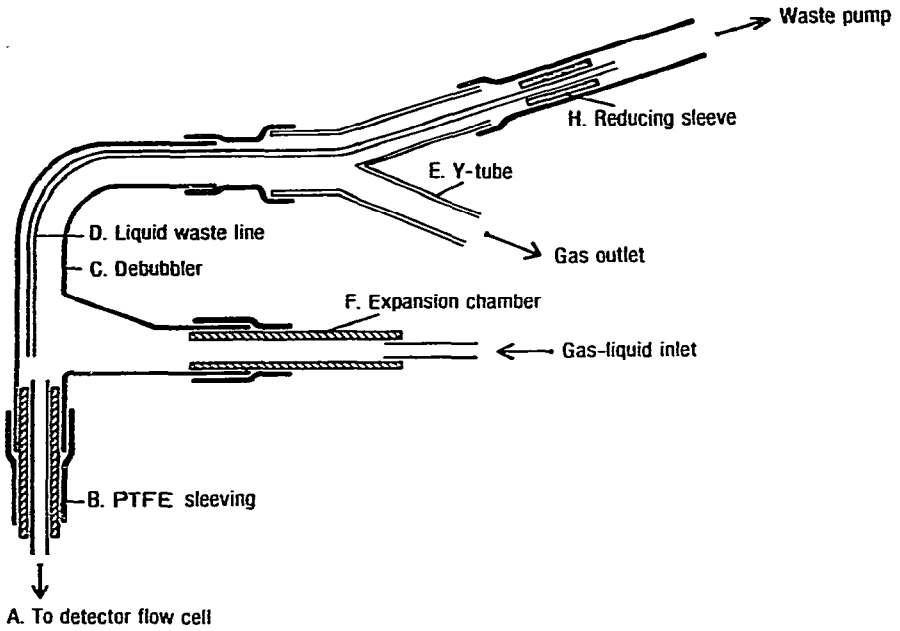

Fig. 2. The debubbler. A = Inlet tubing to detector flow cell, PTFE tubing 0.015 in. I.D. B = Heat-shrinkable PTFE sleeving sealing tubing A into C. C = Technicon glass C5 "T" connector with debubbler. D = Liquid waste line, PTFE tubing 0.034 in. I.D. E = Y tube for separating liquid waste line from carrier gas. F = 1 × 0.059 in. I.D. PTFE tubing. H = Reducing sleeve to seal waste line to pump tubing.

at room temperature under a gentle stream of nitrogen. The residue was taken up in 25 μl of isooctane, of which 5 μl was then injected into the GLC column.

RESULTS

As noted previously, methyl *p*-chlorophenoxyisobutyrate (Me-CPIB), methyl *p*-chlorophenoxyproprionate (Me-CPP) and methyl *p*-chlorophenoxyacetate (Me-CPA) had retention times on the EGA column similar to those of the methyl esters of long-chain fatty acids (Fig. 3). Under the conditions used here (175°C, EGA), Me-CPIB eluted between methyl myristate (14:0) and methyl palmitate (16:0), Me-CPP just after and incompletely resolved from (16:0), and Me-CPA just prior to methyl stearate (18:0). As expected, the UV detector (Fig. 3, lower record) ignored the elution of the fatty acid esters completely. The relative responses of the UV and FID detectors to the three chlorophenoxy compounds were different, reflecting the differences in the molecular properties to which the detectors respond.

The three compounds eluted from the OV-17 column well before the long-chain fatty acid methyl esters. In analyses of serum samples in which the lipids were extracted along with the free chlorophenoxy acids, the long-chain fatty acid esters in the sample being analyzed did not interfere in the identification, even on the FID record. However, when several samples were analyzed sequentially, fatty acid esters from previous samples emerged at somewhat unpredictable times during the analyses and distorted the FID record (Fig. 4). These late-emerging peaks were identifiable as

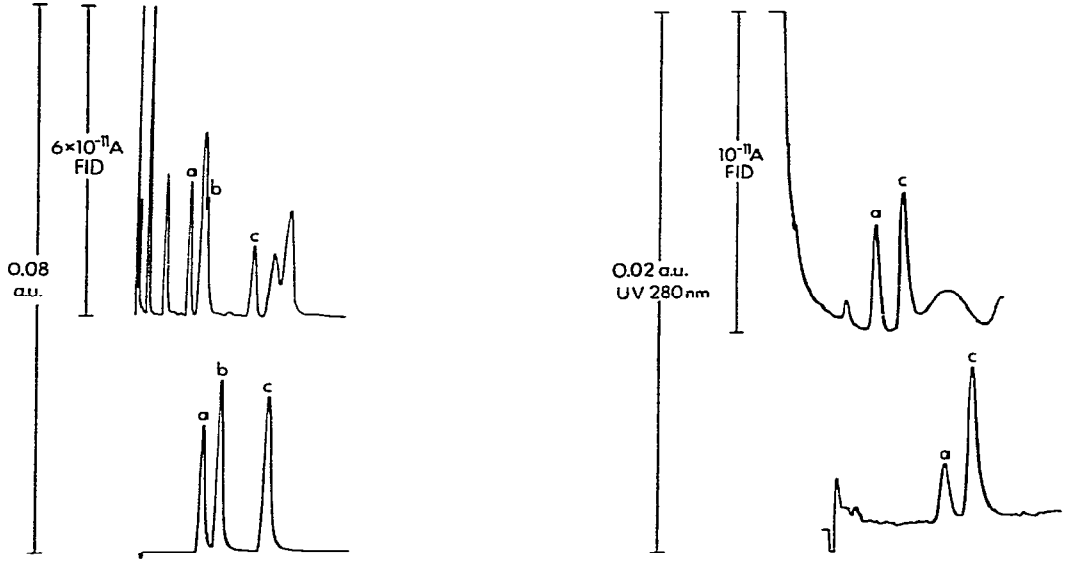

Fig. 3. FID (upper) and UV (lower) records of a sample containing 2 μl of 0.75% (v/v) Me-CPIB (a), Me-CPP (b), Me-CPA (c) in isooctane and 2 μl of 1% solution of the methyl esters of ivory soap in isooctane. Chromatographic conditions: 10% EGA on Chromosorb W HP; column temperature, 175°C; nitrogen flow-rate, 30 ml/min.

Fig. 4. FID (upper) and UV (lower) records of a serum extract containing CPIB (a) and CPA (c) at concentrations of 50 μg/ml each. Chromatographic conditions: 3% OV-17; column temperature 125°C; nitrogen flow-rate 27 ml/min. Analysis time shown: 20 min.

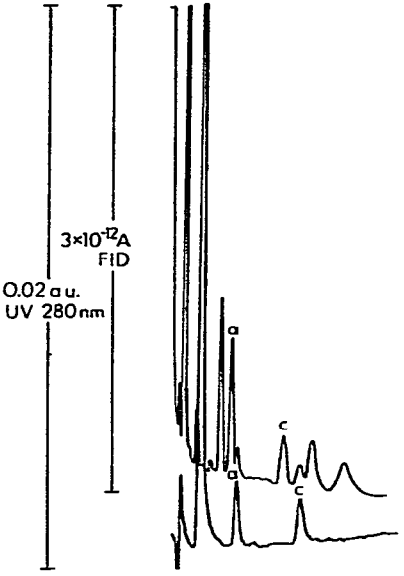

Fig. 5. FID and UV records of a typical serum extract containing CPIB (a) and CPA (c) at concentrations of 50 μg/ml each. Chromatographic conditions: 10% EGA; column temperature, 175°C; nitrogen flow-rate, 30 ml/min. Analysis time shown: 19 min.

not being part of the sample being analyzed because of their appreciably greater peak widths. In isothermal analyses using the FID with these relatively non-polar columns it was found advisable either to limit the rate of repetition of analyses, or, preferably, to program the temperature at the conclusion of the analysis to clear compounds eluting later from the column. As shown, the UV detector again ignored the elution of these fatty acid esters.

Virtually the same effect was observed in analyses of extracts of serum samples to which CPIB and the internal standard, CPA, had been added (Fig. 5). In sixteen samples taken from hospitalized patients known to be taking a variety of medications, no compounds that interfered were observed, indicating that no compounds that absorbed at 280 nm were eluted with retention times similar to those of the Clofibrate and similar compounds. One of the samples showed an unidentified compound eluting immediately after the solvent that absorbed at 280 nm (Fig. 5).

DISCUSSION

Sensitivity of detection of CPIB and the similar compounds used as internal standards was as predicted from the extinction coefficients of the derivatives and the flow-rate. The noise level was reduced by jacketing the scrubber coil with heat-exchanger tubing, and suppressing pulsations in flow from the pulling pump, and fastening 10 in. of the inlet tubing to the detector cell housing. Peak-to-peak noise over a 15-min interval was 0.0005 a.u. At a flow-rate of the scrubbing solvent of 0.46 ml/min, a 0.14-μg injection of Me-CPIB gave a signal-to-noise ratio of five. We predicted a ten-fold increase in sensitivity by monitoring at 230 nm, where the absorbance of the CPIB was higher, but could not predict the noise.

Each of the components of the detection system can contribute to peak broadening and loss of resolution. In the work reported here, the two most important sources of peak broadening were the debubbler and the detector inlet tubing, requiring that most care be taken in the operation of these components. The design of debubbler detailed in Fig. 2 limits mixing volume to that determined by the height of the vacuum waste line (d_1) above the plane of the detector inlet tubing. To avoid incomplete debubbling when using such a small volume, one must insure little or no fluctuation in the solvent flow-rate from the scrubbing coil into the debubbler. The combination of a constant-pressure solvent-delivery system and reduction of pressure surges caused by partial vaporization of the solvent into the scrubbing coil was found helpful. We performed the Clofibrate assay using the same approach others had used previously[5-8]: acidification, extraction into an organic phase, esterification with methanol, and GLC on a semi-polar column. We chose this assay to explore the utility of the UV detector in therapeutic drug monitoring with GLC because it presented analytical difficulties, albeit mild, when done with conventional GLC detectors, and because the compounds were known to absorb in the ultraviolet. The UV detector offered advantages that were largely as predicted. It seems reasonable to predict that lipid-soluble, UV-absorbing materials of the kind typified by CPIB, including drugs and their metabolites, could be detected in lipid extracts of tissues with greater effective sensitivity than with other GLC detectors, since the specificity of detection permits larger samples to be injected into the column.

ACKNOWLEDGEMENT

This work was supported by Grant GM 19478 from the National Institute of General Medical Sciences, National Institutes of Health.

REFERENCES

1 M. Bowman and M. Beroza, *Anal. Chem.*, 40 (1968) 535.
2 T. Tsuda and D. Ishii, *J. Chromatogr.*, 96 (1974) 171.
3 F. Chow and A. Karmen, *Clin. Chem.*, 26 (1980) 1480.
4 A. Karmen, S. Lam, G. Cummings and F. Chow, *J. Chromatogr.*, 199 (1980) 113.
5 G. Houin, J. J. Thébault, P. d'Athis, J.-P. Tillement and J.-L. Beaumont, *Eur. J. Clin. Pharmacol.*, 8 (1975) 443.
6 E. M. Faed and E. G. McQueen, *Pharmacology*, 12 (1974) 144.
7 T. C. Cuong and A. Tuong, *J. Chromatogr.*, 106 (1975) 97.
8 A. Karmen and H. Haut, *Biochem. Med.*, 12 (1975) 154.