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ESTERIFICATION OF TRIFLUOROACETIC ACID WITH PHENYLDIAZOMETHANE FOR QUANTITATIVE GAS CHROMATOGRAPHIC ANALYSIS

METHODS INVOLVING SEPARATION FROM BIOLOGICAL MATERIALS

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SUMMARY

Trifluoroacetic acid in biological materials has been quantitatively determined by gas chromatography. Benzyl trifluoroacetate has been prepared by the reaction of the acid with phenyldiazomethane, and has been successfully analyzed by gas chromatography without any interference from other peaks. The procedure has been used to determine trifluoroacetic acid in microsomal suspension incubated with halothane, a gaseous anaesthetic.

INTRODUCTION

During an investigation of the biological oxidation of halothane, a gaseous anaesthetic, to trifluoroacetic acid (TFA), it was necessary to determine quantitatively the small amount of TFA present *in vitro*[1]. The quantitative gas chromatographic (GC) determination of such low-molecular-weight and highly polar carboxylic acids is very difficult due to their strong adsorption to the stationary phases[2], and the extraction of such polar short-chain carboxylic acids from aqueous solution by use of organic solvents is laborious because of the volatility and the unfavourable distribution coefficients[2]. The extraction of TFA from biological materials by use of organic solvents is also difficult, because the acid is intimately bound to large cell fragments in cell membranes, microsomes and mitochondria[3].

Attempts have therefore been made to find a less polar derivative than TFA which could be prepared quantitatively and which has sufficiently low volatility to minimize the loss in handling and concentrating prior to chromatography. In these

experiments, TFA was esterified with phenyldiazomethane (PDM) both in diethyl ether and in deproteinized hepatic microsomal solution and has been successfully applied to the gas chromatograph. The recovery rates of TFA in deproteinized hepatic microsomal solution have been examined by the use of the above method under various experimental conditions. These methods have also been extended to the quantitative determination of TFA, which is proposed as the end product of biological oxidation of halothane by rabbit hepatic microsomes.

EXPERIMENTAL

Materials

TFA, trifluoroacetic anhydride (TFAA), benzyl alcohol, benzylamine, *p*-toluenesulphonyl chloride and anisole were purchased from Tokyo Kasei Kogyo, Tokyo, Japan. Reduced nicotinamide-adenine dinucleotide phosphate (NADPH), glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from E. Merck Japan, Tokyo, Japan. Halothane containing 0.01 % thymol was a gift from Hoechst Japan, Tokyo, Japan; it was distilled and used throughout the experiments. 3 % OV-17 on Gas-Chrom Q was purchased from Nippon Chromato Kogyo, Tokyo, Japan. Other chemicals were obtained from commercial sources.

Phenyldiazomethane (PDM). As illustrated in Fig. 1, PDM was prepared according to a series of reactions. N-Benzyl-*p*-toluenesulphonamide was prepared by the method of Hinsberg[4]. N-Benzyl-N-nitroso-*p*-toluenesulphonamide was prepared by the method of Overberger and Anselme[5]. Both products were recrystallized from ethanol, and were characterized by their IR spectra and melting points.

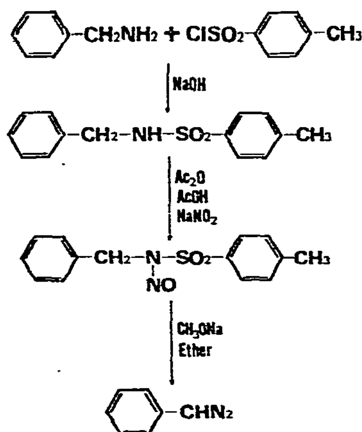
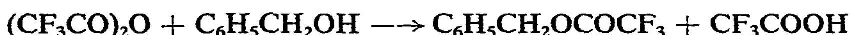


Fig. 1. Synthesis of phenyldiazomethane.

PDM was prepared according to the procedure of Overberger and Anselme[5] and that of Klemm *et al.*[2] with minor modification. Over a period of 1 h, 87 g (0.3 mole) of N-benzyl-N-nitroso-*p*-toluenesulphonamide was gradually added to a mixture of 17.2 g (0.3 mole) of sodium methoxide, 100 ml of absolute methanol and 540 ml of absolute diethyl ether, stirring the reactants vigorouslv at 5°. The reaction

mixture was then heated under reflux for 30 min. After cooling of the mixture, 1 l of ice-cold water was added to dissolve the salts, and the aqueous layer was discarded. The solution of PDM in diethyl ether was washed twice with additional portions of ice-cold water and dried over anhydrous magnesium sulphate. The resulting solution was cooled at −30° for 45 min, and then filtered in order to remove the changed reactants and by-products. Further purification of this solution of PDM in diethyl ether was not carried out because several explosions have been reported in the preparation of PDM[6] and the solution has a satisfactory purity for the preparation of the benzyl ester of TFA. The esterification of TFA was carried out immediately after the preparation of PDM.

Benzyl trifluoroacetate (BTFA) from TFAA. BTFA was prepared from trifluoroacetic anhydride and benzyl alcohol according to the scheme:



$$(CF_3CO)_2O + C_6H_5CH_2OH \longrightarrow C_6H_5CH_2OCOCF_3 + CF_3COOH$$

The BTFA product was characterized by measurement of its boiling point (173–174°/760 mmHg)[7] and by use of gas chromatography–mass spectrometry (GC–MS).

Procedure

The investigations were carried out by use of a hepatic microsomal suspension [5 mg protein per ml, in 0.1 *M* potassium phosphate buffer (pH 7.4)] obtained from rabbit liver which was pre-treated with phenobarbital as described previously. In order to examine the recovery rates of TFA from biological fluids without interference with the proteins, the deproteinized hepatic microsomal solution was prepared as follows and was used throughout the experiments. The hepatic microsomal suspension was deproteinized by the addition of concentrated HCl, followed by centrifugation at 9000 *g* for 10 min. The supernatant was adjusted to pH 7.4 with 10 *N* NaOH and was used as the microsomal solution.

Preparation of BTFA

Freshly prepared PDM in diethyl ether was gradually added to a solution of TFA in diethyl ether [5 μl (65 nmole) of TFA in 100 ml of ether], with vigorous stirring at room temperature. The PDM was added until its red colour persisted. Esterification was accompanied by the disappearance of the colour. After stirring overnight at room temperature, the mixture was concentrated to *ca.* 1 ml in a water-bath, and then kept at −20° for over 12 h. The resulting white-yellow crystals were filtered off and the remaining solution was then made up to 5 ml with additional portions of diethyl ether. 3 μl of this solution was used for GC and GC–MS. Anisole was used as an internal standard in gas chromatography.

Separation of TFA from biological materials

(1) *Solvent extraction.* 5 μl (65 nmole) of TFA was added to 10 ml of deproteinized hepatic microsomal solution. After stirring for 30 min, the mixture was adjusted to between pH 0.5 and 7.0 with concentrated HCl or 10 *N* NaOH. The TFA was extracted 3–9 times with 10 ml of diethyl ether. The ether phases were combined and dried over anhydrous magnesium sulphate. PDM in diethyl ether was then added directly in order to esterify the TFA as described above.

(2) *Direct esterification of TFA in biological materials.* 5 μl (65 nmole) of TFA was added to 10 ml of deproteinized hepatic microsomal solution and the mixture was adjusted to between pH 1.0 and 7.0 with concentrated HCl or 10 *N* NaOH. PDM in diethyl ether was added directly to the mixture until the red colour of PDM persisted and the pH was 7.0. After stirring overnight at room temperature, the ether phase was separated and dried over anhydrous magnesium sulphate. This solution was concentrated, made up to 5 ml and then used for GC and GC–MS as described above.

(3) *Protein binding of TFA.* 5 μl (65 nmole) of TFA was added to the hepatic microsomal suspension (5 mg protein per ml, 10 ml, pH 7.4). After stirring for 30 min at room temperature, the mixture was adjusted to pH 0.5 with concentrated HCl. The resulting denatured protein was removed by centrifugation at 9000 *g* for 10 min. 10 ml of diethyl ether were then added to the supernatant, and the TFA was extracted 9 times with additional portions of diethyl ether. The ether phases were treated with PDM as described and used for GC and GC–MS.

Incubation of the hepatic microsomal suspension with halothane

Microsomal suspension (50 ml) was incubated in a water-bath at 25° and 4% halothane vapour was insufflated with air at a flow-rate of 4 l/min. After pre-incubation for 30 min in order to equilibrate the halothane with the medium, a reaction was initiated by the addition of 1.0 m*M* reduced nicotinamide-adenine dinucleotide phosphate (NADPH). Glucose-6-phosphate (5 m*M*) and glucose-6-phosphate dehydrogenase (1 ng/ml) were also added as a NADPH regenerating system. The incubation was continued for 30–180 min. The reaction was terminated by the addition of concentrated HCl, and the mixture was centrifuged at 9000 *g* for 10 min. The supernatant was adjusted to pH 0.5 with concentrated HCl and extracted 9 times with 10 ml of diethyl ether. The ether phases were combined, treated with PDM, concentrated and then made up to 5 ml. 3 μl of this sample was injected into the gas chromatograph.

Gas chromatography and gas chromatography–mass spectrometry

The GC and GC–MS analyses were made on a JEOL Model JGC-1100 gas chromatograph equipped with a flame ionization detector, and on a JMS-01SG-2 double-focusing mass spectrometer connected to a JGC-20K gas chromatograph via a two-stage jet-type separator. A glass column (3 m × 3 mm I.D.) packed with 3% OV-17 on Gas-Chrom Q (60–80 mesh) was used. The operation temperatures of GC were 110° (column), 130° (injector) and 150° (detector). Nitrogen was used as the carrier gas. The GC conditions of GC–MS were the same as above, except that helium was used as the carrier gas, the separator and ion-source temperatures were 200°, the accelerating voltage was 8 kV and the electron energy was 75 eV.

The extent of esterification of TFA by PDM and the recovery rates of TFA from biological materials were calculated from a calibration graph constructed from the gas chromatogram of BTFA synthesized from TFAA.

RESULTS

As illustrated in Fig. 2A, the gas chromatogram of TFA treated with PDM showed 9 peaks when TFA was esterified in diethyl ether. The retention time and the mass spectrum of peak III (Fig. 3) were identical with those of BTFA synthesized

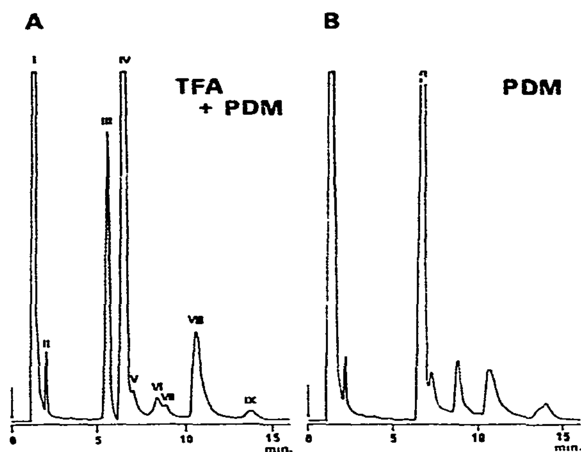


Fig. 2. Gas chromatograms of TFA treated with PDM in diethyl ether (A), and of the ether solution of PDM (B).

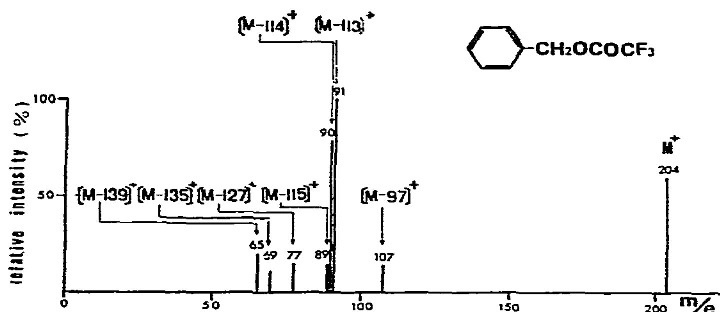


Fig. 3. Mass spectrum of peak III.

from TFAA. The molecular ion of BTFA at *m/e* 204 was clearly observed. The fragment ions at *m/e* 107, 91, 90, 77 and 69 were identical to those of authentic BTFA, and correspond to the ions $[C_6H_5CH_2O]^+$, $[C_7H_7]^+$, $[C_6H_4CH_2]^+$, $[C_6H_5]^+$ and $[CF_3]^+$, respectively[8]. The ion at *m/e* 65 was formed by loss of acetylene from the tropylium ion at *m/e* 91, and the ion at *m/e* 89 was formed by loss of a hydrogen atom from the ion at *m/e* 90 (ref. 8). From these results, peak III is identified as due to BTFA. The extents of esterification of TFA with PDM were between 91 and 95% ($n = 5$) as calculated from the calibration graph constructed from the gas chromatogram of authentic BTFA.

Peak I showed the same retention time as that of diethyl ether. From analyses of the mass spectra, peaks IV, VI and VIII were identified as $C_6H_5CH_2OCH_3$, C_6H_5CN and $C_6H_5CH_2OH$, respectively (Figs. 4, 5 and 6). The remaining peaks in the gas chromatogram could not be identified from mass spectral analyses. These peaks seem to be by-products of the synthesis of PDM, because they were also detected in a gas chromatogram of the diethyl ether solution of PDM (Fig. 2B).

The overall recovery of TFA, added to the deproteinized hepatic microsomal solution, is shown in Figs. 7 and 8. 83 $\pm$ 3% TFA was recovered from the depro-

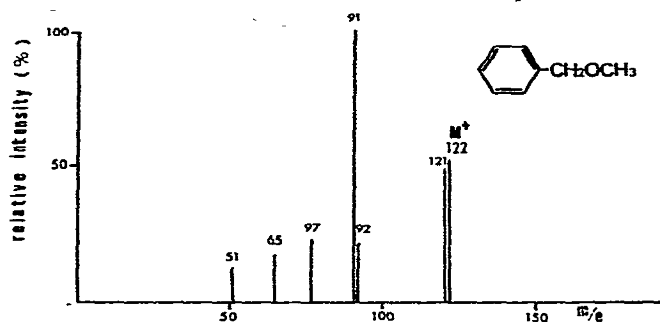


Fig. 4. Mass spectrum of peak IV.

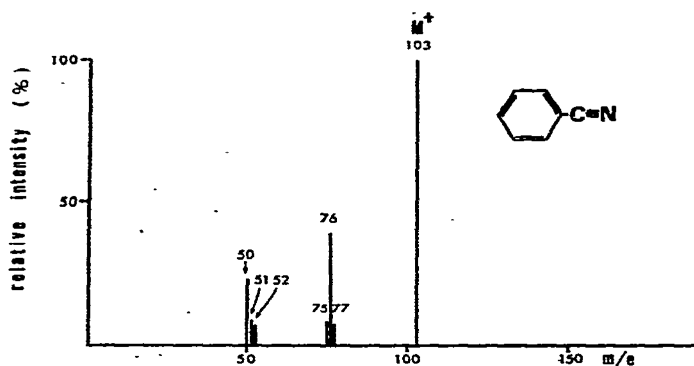


Fig. 5. Mass spectrum of peak VI.

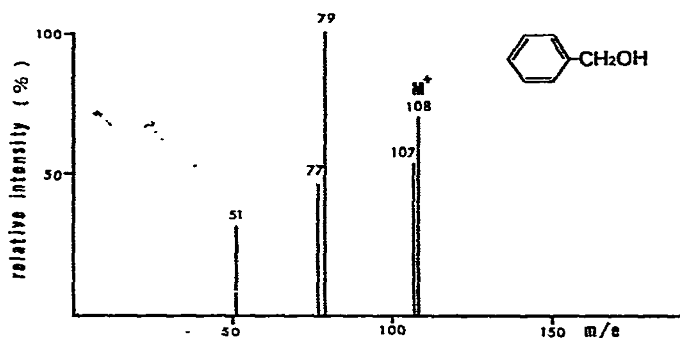


Fig. 6. Mass spectrum of peak VIII.

teinized hepatic microsomal solution (pH 1.0) when the acid was directly esterified with PDM (Fig. 7B). Direct esterification of TFA takes place in the range pH 1.0–7.0. The recoveries were 90 and 93%, respectively, when TFA was extracted over 5 times with diethyl ether at pH 0.5 and was treated with PDM without concentrating the ether phases as described above (Fig. 8). TFA could not be recovered when the pH of the solution was greater than 5.0 (Fig. 7A).

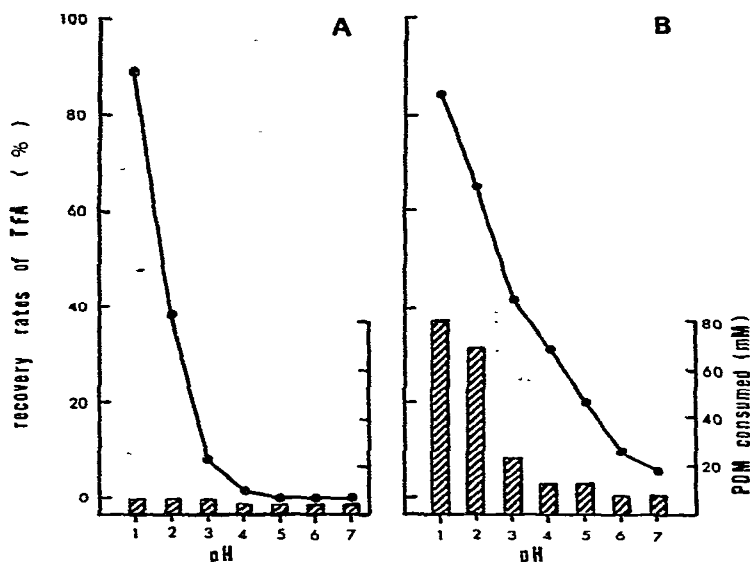


Fig. 7. Recovery of TFA from deproteinized hepatic microsomal solution. TFA was extracted 9 times with diethyl ether, and esterified with PDM without concentrating the ether phases (A), or directly esterified with PDM in deproteinized hepatic microsomal solution (B).

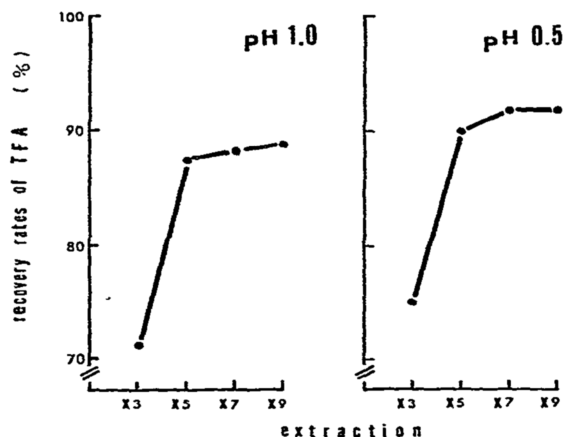


Fig. 8. Recovery of TFA from deproteinized hepatic microsomal solution. TFA was extracted 3–9 times with diethyl ether and esterified with PDM without concentrating the ether phase.

The consumption of PDM was greater than 80 mmoles when TFA was directly esterified in hepatic microsomal deproteinized solution (pH 1.0), while the consumption of PDM was less than 5 mmoles when TFA was extracted and treated with PDM (Fig. 7A). The recoveries of TFA, added to the microsomal suspension, always remained constant between 75 and 80%. Consequently, the amounts of TFA, binding to hepatic microsomal proteins, were in the range 20–25%.

TFA was quantitatively determined in an acidic extract of an incubation medium containing microsomes, NADPH, oxygen and halothane (Fig. 9). The time course of the formation of TFA in rabbit hepatic microsomes is illustrated in Fig. 10.

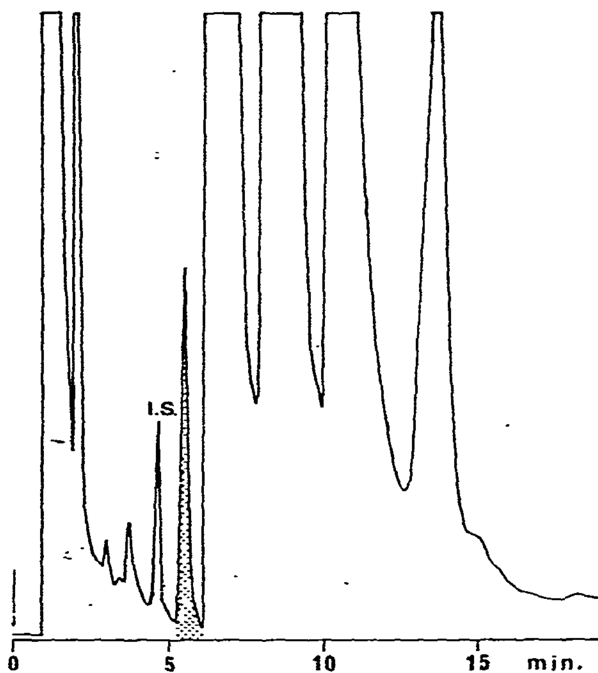


Fig. 9. Gas chromatogram of PDM-treated acidic extract of an incubation medium containing microsomes, NADPH, oxygen and halothane. I.S. = Anisole internal standard.

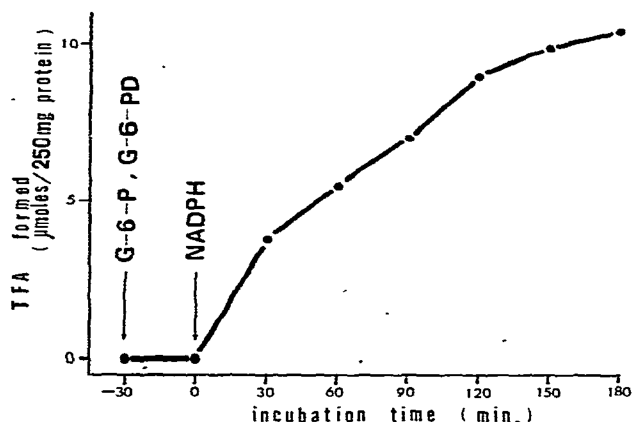


Fig. 10. Time course of the formation of TFA from halothane in rabbit hepatic microsomal suspension. G-6-P = Glucose-6-phosphate; G-6-PD = glucose-6-phosphate dehydrogenase.

DISCUSSION

For long-chain carboxylic acids, the quantitative GC analysis of their methyl esters is generally used[9]. However, for short-chain carboxylic acids, a comparable standard method has not been established.

TFA is a volatile (b.p. 71.78°) low-molecular-weight (mol. wt. 114) carboxylic acid. The GC analysis of TFA is complicated due to its strong adsorption to the stationary phases and to other problems[1]. This problem was overcome when the methyl ester of TFA was prepared by use of N,N'-dimethylformamide dimethyl acetal[1]. Quantitative determination of TFA from biological materials is difficult because of the extreme volatility, greater than that of TFA itself, of the methyl ester of TFA.

The boiling point of BTFA is in the range 173–174° at 760 mmHg, as described by Weygand and Leising[7]. Thus the difficulties which arise from the extreme volatility of the methyl ester can be largely avoided by use of the benzyl ester. Indeed, in the present experiments solutions of BTFA in dilute diethyl ether were prepared quantitatively by use of PDM and were successfully separated on the gas chromatograph without any adsorption to the stationary phases (Fig. 2A). The extents of esterification of TFA with PDM were satisfactory and were between 91 and 95% ($n = 5$).

The second problem which arises from the unfavourable distribution coefficients of TFA is the recovery of TFA from biological materials on extraction with organic solvents. We examined the direct esterification of TFA with PDM in deproteinized hepatic microsomal solution. The overall recovery of TFA was 83 ± 3% when the solution was adjusted to pH 1.0 and a large quantity of PDM was consumed (Fig. 7B). On the other hand the overall recovery of TFA was in the range 90–93% when the acid was extracted 5–9 times with diethyl ether at pH 0.5 and was treated with PDM without concentrating the ether phases (Fig. 8). Less than 5 mmoles of PDM were consumed in this procedure (Fig. 7A). This method is therefore better than that of direct esterification of TFA in biological materials. Also direct esterification of TFA with PDM in biological materials occurred up to pH 7.0, and the consumption of PDM was less than 10 mmoles when the pH of the solution was greater than 4.0 (Fig. 7B). From these results, it is estimated that direct esterification with PDM will be useful for GC determinations of very small quantities of low-molecular-weight carboxylic acids, such as acetic acid, because their pK_a values are higher than that of TFA (pK_a 0.23).

A third problem is the binding of TFA to large cell fragments present in cell membranes, microsomes and mitochondria. The extent of binding of TFA to hepatic microsomal proteins was calculated to be between 20 and 25% when TFA was added to the hepatic microsomal suspension, because the recovery of TFA, added to the microsomal suspension, always remained constant beteeen 75 and 80%.

From all these results, it is concluded that TFA in hepatic microsomal suspension can be quantitatively determined by gas chromatography. The method is also successful in the quantitative determination of TFA, the oxidative metabolite of halothane, in experiments *in vitro* (Figs. 9 and 10).

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