Journal of Chromatography, 171 (1979) 279–283

CHROM. 11,558

GAS CHROMATOGRAPHIC DETERMINATION OF METHYLCYCLOPENTADIENYLMANGANESETRICARBONYL IN BIOLOGICAL TISSUES AND FLUIDS

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(Received October 24th, 1978)

SUMMARY

The procedure described for quantitating methylcyclopentadienylmanganesetricarbonyl (MMT, a fuel additive) in small samples of biological fluids and tissues is based on extracting the MMT into hexane containing biphenyl as internal standard, followed by gas chromatographic analysis. With flame ionisation detection, as little as 1–2 ppm of MMT in tissue can be determined relatively easily. The method is also applicable to *in vitro* investigations of MMT metabolism, and it has been used to show that the enzymic oxidation of MMT by rat-liver microsomes is a cytochrome P-450-dependent process.

INTRODUCTION

An organometallic derivative of manganese, methylcyclopentadienylmanganesetricarbonyl (h^5-$CH_3C_5H_4Mn(CO)_3$; MMT), is currently used as a substitute for tetraethyl-lead to improve the octane rating of "no-lead" gasoline[1,2]. In connection with an investigation of the metabolism and toxicological properties of MMT in mammals[3,4], the need arose for a sensitive, specific and rapid method for determining MMT in small biological samples of various kinds. Although atomic absorption spectroscopy (flame atomization mode) has been used to determine MMT in JP-4 jet fuel[5], this method lacks the desired sensitivity and convenience for biological studies. Our initial attempts to achieve better sensitivity by using atomic absorption with a carbon-rod atomizer (Varian CRA-90) were frustrated by the appreciable volatility of MMT, and by our inability to devise chemical methods for converting it with reproducible efficiency into non-volatile inorganic forms of manganese more suitable for analysis by flameless atomic-absorption spectroscopy. On the other hand, the volatility of MMT suggested that it might be amenable to gas chromatographic determination. This proved to be so, and we report herein a gas chromatographic

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method, with flame ionization detection, for determining MMT in the low ppm range and above in biological fluids and tissues.

MATERIALS AND METHODS

MMT was obtained from Strem Chemicals (Newburyport, Mass., U.S.A.) as an orange liquid. Pure MMT, a yellow liquid, was obtained by distillation at reduced pressure (b.p. 67°, 1 Torr), through a 40 × 1 cm Vigreux column. Since MMT is light-sensitive[3,6], the distillation was carried out under subdued lighting with most of the apparatus wrapped in aluminum foil. Immediately after distillation, aliquots of the MMT were sealed in glass 2-ml ampoules and stored in a dark box at −15°; after 8 months, no decomposition was detectable. Biphenyl was obtained from Eastman and used without purification. Skelly B solvent (hexanes) was distilled through a 60-cm Vigreux column (b.p. 65–69°) before use. Other reagents were of analytical grade.

Extraction procedure

Blood or serum samples were diluted, and tissue samples homogenized, with 4 volumes of distilled water, all operations being carried out under subdued room lighting. A 1.0-ml aliquot of the resulting preparation was transferred to a 13 × 100mm culture tube fitted with a PTFE-lined screw-cap closure. At this point, if "spiked" standards rather than unknowns were being prepared, a 2–20-μl aliquot of a solution of 5000 ppm (w/v) of MMT in acetone was added from a syringe, and the tube was vortexed gently to achieve thorough mixing. A 2.0-ml aliquot of a 50-ppm solution of biphenyl in hexanes was then added, and the tube was capped and vortexed for 15–20 sec. Trichloroacetic acid solution (30%, 0.5 ml) was then added and the vortexing step was repeated. Centrifugation at *ca.* 200 *g* for 5 min separated the emulsion into a hard bottom pellet, a clear aqueous layer, a firm interfacial layer and a clear upper layer of organic solvent that could easily be removed by pipette or syringe for analysis. The hexane extracts were stored in a darkened container until analyzed, usually within 24 h.

Gas chromatography

Equipment. A Varian Model 1400 gas chromatograph equipped with a flame ionization detector was used with one of two columns. Column A (6 ft. × ⅛ in.) was of stainless steel and was packed with 10% of DC-550 silicone oil on 100–120 mesh Chromosorb W AW DCMS. Nitrogen was used as carrier gas at a flow-rate of 40 ml/min, and the operating temperatures were: injector, 200°; oven, 180°; and detector, 200°. Under these conditions, the retention times of MMT and biphenyl were 6.6 and 12.2 min, respectively. Column B (6 ft. × 2 mm I.D.) was of glass and was packed with 3% of DC-710 silicone oil (similar to OV-17) on 100–120 mesh Chromosorb W AW DCMS. The carrier gas was nitrogen (40 ml/min), and the operating temperatures were: injector, 200°; oven, 130°; and detector, 220°. Under these conditions, the retention times of MMT and biphenyl were 3.8 and 8.2 min, respectively.

Data handling. The output signal from the chromatograph was recorded on a Heathkit recorder in parallel with a Varian CDS-111 computing digital integrator.

Calibration graphs and recovery. Response factors were determined from "100% recovery" samples prepared by combining 2–20 μl of 5000-ppm MMT solution in acetone with 2.0 ml of 50-ppm biphenyl solution in hexanes, and analyzing this mixture directly on the gas chromatograph. The same solutions were also used in various combinations to calibrate and to double check the integrator, since the latter automatically converted the relative peak areas of the biphenyl internal standard and MMT into concentrations of MMT in the sample. Recoveries from biological material were then determined by "spiking" the sample homogenates with MMT and analyzing them as described above. In order to verify that the MMT did not decompose under the chromatographic conditions, a stainless-steel tee-piece and needle valve were installed between the end of the column and the detector to act as an adjustable effluent-stream splitter. In this way, material emerging under the MMT peak was collected in a glass tube cooled externally with a stream of cold carbon dioxide vapour. A yellowish liquid condensed and was identified as MMT by its odor, its thin-layer chromatographic properties [R_F 0.59–0.69 on silica, with ethyl acetate–hexane (5:95) as mobile phase] and its infrared spectrum in chloroform solution.

RESULTS AND DISCUSSION

Analysis of solutions containing 50 ppm of biphenyl and 5–250 ppm of MMT gave an excellent correspondence between observed and expected concentrations of MMT when the instrument was calibrated with a 50 ppm MMT–50 ppm biphenyl standard (column A, 3-μl injections). A plot of these data over a 50-fold concentration range gave a straight line (slope 1.021; intercept, −0.677 ppm; $r = 0.9998$), and repetitive analyses of samples in this range usually gave relative standard deviations much less than $\pm$ 1%. Over a period of 3 weeks, during which the calibration solution was stored at room temperature in an amber-glass bottle, the relative response factor determined by the integrator for MMT *vs.* biphenyl remained almost constant at 4.382 ± 0.156 ($n = 19$). However, because of non-volatile combustion products from the MMT, it was necessary to clean the detector assembly thoroughly at monthly intervals to maintain the optimum performance and sensitivity.

Since the integrator performed best when the ratio of analyte to internal standard was in the range 0.1–10, it was necessary to use a lower concentration of internal standard in order to extend the useful range of the assay below 5 ppm of MMT. Thus, a solution of 10 ppm of biphenyl in Skelly B was used, and analyses were performed on Column B (2-μl injections).

For all biological tissues studied, the extraction and recovery of MMT were fairly good. Table I gives the slopes, intercepts, and correlation coefficients for calibration curves prepared for a range of MMT concentrations in diluted serum or homogenates of brain, liver, lung or kidney. The slopes of these calibration plots give the extraction efficiencies. As can be seen, recovery is good, even from brain homogenate, which is often troublesome because of its high lipid content. This is no doubt a reflection of the extremely apolar and lipophilic nature of MMT. In contrast to those for diluted whole blood, results for undiluted whole blood were much less predictable, probably because of problems associated with its viscosity and intense coagulation after addition of trichloroacetic acid.

TABLE I

EXTRACTION EFFICIENCY FOR MMT FROM VARIOUS BIOLOGICAL MATRICES

Tissue/homogenate	*Range of MMT concn. (ppm)*	*GLC column*	*Slope**	*Intercept (ppm)*	*r*
Brain (1:4)	10 – 50	A	0.945	−1.75	0.9992
Liver (1:4)	10 – 50	A	0.975	−2.85	0.9875
Blood (1:4)	10 – 50	A	0.935	−2.67	0.9989
Serum (1:3)	10 –100	A	1.027	−3.6	0.9928
Lung (1:4)	10 –100	A	0.967	−2.9	0.9990
Lung (1:4)	0.5– 16	B	0.996	−0.04	0.9997
Liver (1:4)	0.5– 16	B	0.997	−0.05	0.9998
Kidney (1:4)	0.5– 16	B	0.995	−0.05	0.9979

* Slope of plot of observed *vs.* calculated MMT concentration; the extraction efficiency (%) is obtained by multiplying the slope by 100.

APPLICATION

As an example of the utility of the proposed method, it was used to investigate the bio-transformation of MMT into polar metabolites by rat-liver microsomes. The microsomes were prepared by calcium-ion precipitation[7] and were supplemented by a standard NADPH-generating system. Incubations were conducted in 16 × 100-mm culture tubes under air in subdued light at 33° and pH 7.4. Each mixture contained 0.5 ml of microsomes (6–8 mg of protein per ml), 50 μl of NADPH-generating system (NADPH-GS), and sufficient MMT in 25 μl of PEG-400 to give a final concentration of 0.5 m*M*. Reactions were stopped by adding 0.5 ml of saturated sodium chloride solution, and the mixtures were extracted with 50-ppm biphenyl solution in hexanes as described above, except that the trichloroacetic acid step was omitted.

The results of a series of incubations designed to determine if cytochrome P-450 was important in the biotransformation of MMT are given in Table II. A preliminary study had shown that the reaction proceeded linearly for at least 16 min, so each incubation was carried out for 15 min. Judging from the very high rate of oxidative metabolism observed in the complete microsomal system, MMT is indeed a very good substrate for microsomal mixed-function oxidases, at least by comparison

TABLE II

BIO-TRANSFORMATION OF MMT *IN VITRO* BY RAT-LIVER MICROSOMES

*Incubation conditions**	*Rate (nmoles/min · mg of protein)***
Complete system, air	13.06 ± 0.36
Boiled control	0.97 ± 0.47
Omit O_2 (100% N_2)	5.51 ± 0.42
Omit NADPH-GS	0.56 ± 0.39
Complete, + N-decylimidazole	1.22 ± 0.15
CO/O_2 (80:20)	4.06 ± 1.17
Complete, induced***	30.52 ± 0.21

* See text.

** Expressed as mean ± standard deviation ($n = 3$).

*** Rats were pre-treated with phenobarbitone (60 mg/kg, i.p.) for 3 days.

with other standard substrates for these enzymes. In experiments with phenobarbital-induced microsomes, over 90% of the MMT (initial concentration 0.5 m*M*) was consumed in 15 min. Table II also shows that the metabolism of MMT requires molecular oxygen and NADPH, and that it is inhibited by both carbon monoxide and N-decylimidazole[8]. Thus, the *in vitro* biotransformation of MMT clearly shows all the hallmarks of a cytochrome P-450-dependent process. Ferrocene, $(C_5H_5)_2Fe$, a similar organometallic π-complex, is also metabolized by cytochrome P-450, part of the hydroxylated product being conjugated and part decomposing with release of iron[9]. We are at present investigating the products of MMT biotransformation in relation to its remarkable toxicity[3,4] relative to that of ferrocene.

ACKNOWLEDGEMENTS

We thank Mr. T. Gillesse for technical assistance with instrumentation, the National Institutes of Health for financial support (ES-01375), and the National Science Foundation for a U.R.P. Fellowship (to S.A.).

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