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Use of radio-gas chromatography and tritium label to optimize derivatization procedures

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ABSTRACT

A sensitive determination of gas chromatographic peak yields by a new radio-gas chromatograph equipped with a synchronized accumulating radioisotope detector is described. Peak yields could be easily determined by the radio-gas chromatograph using [^{3}H]hexadecane or [^{3}H]androstenedione as a standard substance. The usefulness of the determination of peak yields was demonstrated by optimizing the derivatization conditions of 6-keto-prostaglandin $F_{1\alpha}$.

INTRODUCTION

In metabolism studies of trace amounts of biologically active substances, such as prostanoids and steroids, gas chromatography–mass spectrometry (GC–MS) is one of the most reliable and effective methods for determination and identification. However, analyses have been performed without sufficient examination of the optimal analytical conditions, particularly in derivatization steps. We previously reported the determination of GC peak yields, which were defined as the percentage of the amount of an injected substance that reaches the detection system, by using a radio-gas chromatograph (system equipped with a synchronized accumulating radioisotope detector, GC–SARD) and ^{14}C label [1]. The determination enabled the evaluation of derivatization yields and degrees of column adsorption and thermal decomposition during GC processes [2].

Recently, a new GC–SARD system with higher chromatographic resolution than the previous one has been developed in our laboratory, and proved to be effective for highly sensitive analyses of ^{3}H-labelled substances [3,4]. This paper describes the determination of GC peak yields by the combination of the new GC–SARD system and ^{3}H label and its application to the examination of derivatization conditions of 6-keto-prostaglandin $F_{1\alpha}$.

EXPERIMENTAL

Radioactive samples and reagents

n-[1-^{14}C]Hexadecane (2.26 GBq/mmol), *n*-[1-^{3}H]hexadecane (17.9 kBq/

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mmol), [4-^{14}C]androst-4-ene-3,17-dione (1.96 GBq/mmol), [1,2,6,7-^{3}H]androst-4-ene-3,17-dione (3.07 TBq/mmol), 6-keto[5,8,9,11,12,14,15(n)-^{3}H]prostaglandin $F_{1\alpha}$ ([^{3}H]6-keto-$PGF_{1\alpha}$ (5.81 TBq/mmol, radiochemical purity >95%) were purchased from the Radiochemical Centre (Amersham, U.K.), and [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (7.07 TBq/mmol) from Daiichi Kagaku Yakuhin (Tokyo, Japan).The radiochemical purities were more than 97% except for [^{3}H]6-keto-$PGF_{1\alpha}$. [^{14}C]- and [^{3}H]hexadecane were used as cyclohexane solutions, and [^{14}C]- and [^{3}H]androstenedione as toluene–ethanol (9:1, v/v) solutions, and [^{3}H]arachidonic acid and [^{3}H]6-keto-$PGF_{1\alpha}$ as ethanol and acetonitrile–water (9:1, v/v) solutions, respectively.

6-Keto-$PGF_{1\alpha}$ was purchased from Sigma (St. Louis, MO, U.S.A.), N-trimethylsilylimidazole (TMSI), O-methylhydroxylamine hydrochloride (MOX) and pyridine (silylation grade) from Wako (Tokyo, Japan), and *tert.*-butyldimethylsilylimidazole (tBDMSI), dimethyl-*n*-propylsilylimidazole (DMnPSI) and dimethylisopropylsilylimidazole (DMiPSI) from Tokyo Kasei Kogyo (Tokyo, Japan). MOX was used as a 2% pyridine solution. Copper oxide (wire, for elemental analyses, dimensions *ca.* 5 × 1 mm) and iron (sponge, 20–30 mesh) were purchased from Wako and Aloka (Tokyo, Japan), respectively. Iron was stored in a vessel filled with nitrogen. Sephadex LH-20 was purchased from Pharmacia (Uppsala, Sweden). Other reagents were purchased from Wako or Tokyo Kasei Kogyo and were of analytical-reagent grade.

Radio-gas chromatography system

The apparatus [4] previously reported was used as the GC–SARD system equipped with either a packed column (packed GC–SARD) or a capillary column (capillary GC–SARD). A glass column (1 m × 3 mm I.D.) packed with 1.5% OV-1 (Shimalite W, AW-DMCS, 80–100 mesh) and a wide-bore fused-silica capillary column (12 m × 0.53 mm I.D.) coated with non-polar CBP1 (1 μm film thickness) were used. Packed GC–SARD was used only in the experiments on reduction efficiency and counting efficiency. The column effluents were introduced into the SARD without splitting to the mass detector. The oxidation–reduction tube was packed with *ca.* 5 g of copper oxide plus *ca.* 4 g of iron, and heated to *ca.* 800°C, unless otherwise stated. The carrier and counting gases were helium and methane (100 ml/min), respectively. The flow-rate of the carrier gas was 50 ml/min in packed GC–SARD and 5 ml/min in capillary GC–SARD. The sampling time was set at 4 s, and the transit time of gas in the SARD system was 28 s (7 × 4 s) in both packed and capillary GC–SARD [4]. The injection port and column oven temperatures were 200 and 180°C in hexadecane, and 270 and 250°C in androstenedione, respectively. Arachidonic acid and 6-keto-$PGF_{1\alpha}$ were analysed after derivatization, with the column oven temperature programmed from 210 to 290°C at 20°C/min, and from 180 to 290°C at 30°C/min after 1 min of injection, respectively. The injection port temperature was 300°C in each case.

Samples were injected with a Hamilton 10-μl microsyringe or 5-μl zero-dead-

volume microsyringe. The latter microsyringe was used for the calibration of the system by [^{14}C]- and [^{3}H]hexadecane. The sample radioactivity was measured with a liquid scintillation counter (Aloka LSC 903 or 1000).

Radio-gas chromatography of [^{3}H]arachidonic acid

[^{3}H]Arachidonic acid (13.0 GBq/mmol, 8.7 ng) was dissolved in 50 μl of ethanol, and to the solutin in an ice-bath was added 0.5 ml of ethereal diazomethane (CH_2N_2). The solution was allowed to stand at room temperature for 30 min, and then evaporated to dryness under a nitrogen stream. The residue was dissolved in 50 μl of methanol, and 1–4 μl of the solution were injected into the capillary GC–SARD system.

Derivatization of [^{3}H]6-keto-prostaglandin $F_{1\alpha}$

[^{3}H]6-Keto-PGF$_{1\alpha}$ (13.8 GBq/mmol, 100 ng) was dissolved in 50 μl of methanol. To the solution in an ice-bath was added 0.5 ml of ethereal CH_2N_2, and the solution was allowed to stand at room temperature for 30 min. After evaporation of the reaction mixture under a nitrogen stream, the MOX solution (0.1 ml) was added to the residue, and the solution was allowed to stand overnight at room temperature. The reaction mixture was evaporated to dryness under a nitrogen stream, and the resulting residue was dissolved in 50 μl of pyridine. To the solution were added 10 μl of TMSI, tBDMSI, DMiPSI or DMnPSI and the solution allowed to stand at room temperature for 1 h. The reaction mixture was then applied to a Sephadex LH-20 column (60 mm × 5 mm I.D.), which was swollen with chloroform–hexane–methanol (10:10:1, v/v/v) immediately before use, and treated with 3 ml of the solvent used for swelling. The eluate was evaporated to dryness under a nitrogen stream. The residue was dissolved into hexane containing 1% pyridine, and 1–4 μl of the solution were injected into the capillary GC–SARD system.

DMiPSI treatments were also conducted at 25, 8 or −5°C for 10, 20, 30, 45, 60, 90 or 120 min. In the case of 8 and −5°C, the application to the LH-20 column was performed in a cold room (*ca.* 8°C).

RESULTS AND DISCUSSION

Radio-GC with a gas-flow proportional counter is very useful for highly sensitive analyses of ^{3}H-labelled substances, because the counting efficiency of the detector for soft β-rays is extremely high. In the new GC–SARD system, seven identical counter tubes are connected longitudinally in series, and signals from each tube accumulate in synchronization with the travelling speed of the radioactive gas through the counter tubes. Seven-fold counts can thus be obtained and then the precision of measurement theoretically exceeds that of the system using a single counter tube by a factor of $\sqrt{7}$ without a reduction in chromatographic resolution. The combination of the system and the ^{3}H label is considered to be

effective for clear tracing of a very small amount of substances during GC processes.

In GC–SARD, effluents from a GC column are introduced into the oxidation–reduction tube, where ^{14}C- and ^{3}H-labelled substances are converted into $^{14}CO_2$ and $^{3}H_2$, respectively, and the gases, after mixing with the counting gas, are introduced into the gas-flow proportional counters. The relationship between the injected radioactivity (A Bq) and the resulting counts per second under the peak [B cps, *i.e.* radioactive peak intensity (counts)/transit time in the SARD system(s)] is represented by the following expression:

$$B = A \times \text{GC peak yield} \times \text{oxidation efficiency} \times \text{reduction efficiency} \times \text{counting efficiency} \quad (1)$$

GC peak yields include the derivatization yields when a substance is injected after derivatization (without any purification). It has already been demonstrated using ^{14}C-labelled substances that column effluents can be quantitatively oxidized into $^{14}CO_2$ over a wide range of experimental conditions [1]. On the other hand, the reduction efficiency (E_{red}) and the counting efficiency (E_{count}) for ^{3}H are unknown, although E_{count} for ^{14}C is virtually 100% in the present apparatus. The determination of E_{red} and E_{count} is very difficult. Those values are considered to be constant independent of the kinds of substance involved. In any event, the sensitivity and precision of the ^{3}H assay is influenced by the product $E_{red} \cdot E_{count}$. Eqn. 1 is simplified as follows, considering the oxidation efficiency to be 100%:

$$B/A = \text{GC peak yield} \times E_{red} \times E_{count} \quad (2)$$

[^{3}H]Hexadecane is considered to be useful as an index for $E_{red} \cdot E_{count}$ because the GC peak yield of hexadecane is 100% [1]. [^{14}C]Hexadecane (80 Bq) and [^{3}H]hexadecane (60 Bq) were injected into the packed GC–SARD under the usual conditions (see Experimental), and B/A was calculated to be 98.5 ± 1.72 and 62.2 ± 1.08%, respectively. The former B/A by [^{14}C]hexadecane can be used for standardization of the apparatus. The latter B/A agrees with the values reported by Swell [5] and Simpson [6]. The value of $E_{red} \cdot E_{count}$ was calculated to be 63.1% (62.2/0.985), and the reproducibility within the experiments very good. $E_{red} \cdot E_{count}$ fluctuated between experiments with the usual conditions to some extent (60–70%). The reason for the fluctuation may be the change of E_{red} due to the physicochemical state of iron surface. E_{red} may also vary with the temperature of the oxidation–reduction tube and the time during which a substance is in contact with the catalyst. Then, effect of the factors on $E_{red} \cdot E_{count}$ were investigated. Consequently, values of $E_{red} \cdot E_{count}$ ranging from 60 to 70% were obtained with good precision within experiments, under the various temperatures of the oxidation–reduction tube (640–800°C) and flow-rates in the tube (20–80 ml/min).

The relationship between the degree of consumption of catalyst and $E_{red} \cdot$

E_{count} was investigated using [^{3}H]hexadecane. Consequently, $E_{red} \cdot E_{count}$ was almost constant for injections of up to 200 μl.

The accurate estimation of GC peak yields by ^{3}H label is now possible using [^{3}H]hexadecane as a standard substance. The substance is, however, inadequate in capillary columns since an extremely large amount, of the order of milligrams, should be injected owing to the very low specific radioactivity of the commercially available substance, which gives poor chromatographic characteristics. Thus, an alternative standard substance was needed, which can be purchased with high specific radioactivity and analysed without any derivatization for convenience. As a result of a survey, [^{3}H]androstenedione was selected since the GC peak yield of androstenedione was shown to be essentially 100% (97.4/0.979) under the appropriate GC conditions (Fig. 1). The value of $E_{red} \cdot E_{count}$ by [^{3}H]androstenedione was calculated to be 69.0 ± 2.38% according to the eqn. 2 and its day-to-day variation 60–70%, as [^{3}H]hexadecane in packed GC–SARD. [^{3}H]Androstenedione was used as a standard substance for the determination of GC peak yields in capillary GC–SARD in subsequent experiments.

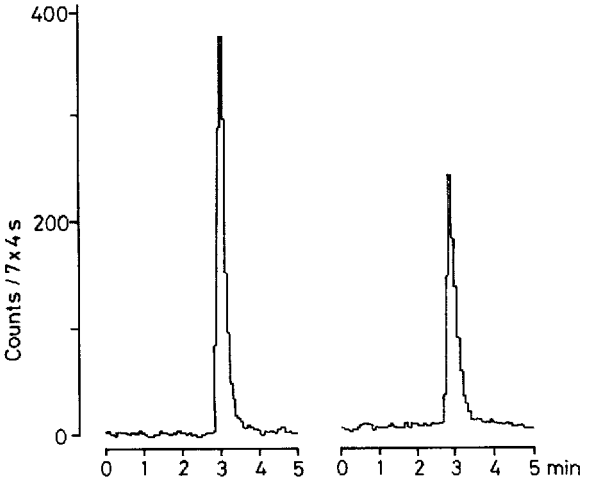

Fig. 1. Radiochromatograms of (left) [^{14}C]- and (right) [^{3}H]androstenedione. Injected radioactivity: [^{14}C]androstenedione (196 MBq/mmol), 71 Bq; [^{3}H]androstenedione (363 MBq/mmol), 92 Bq. B/A (mean ± S.D., $n = 5$, see eqn. 2) = 97.4 ± 1.32% for [^{14}C]- and 67.2 ± 2.32% for [^{3}H]androstenedione. B/A by [^{14}C]hexadecane was 97.9%.

With usual mass detectors, it has been impossible to know whether or not the derivatization is quantitative, and the investigation of the derivatization conditions for micro-amounts has been difficult [7–9]. Now, however, such estimation can be done quite easily by calculating the GC peak yields of the substances under consideration.

First the above method was applied to the derivatization of [^{3}H]arachidonic

acid. [^{3}H]Arachidonic acid was injected into the capillary GC–SARD system after treatment with CH_2N_2, without any purification. As shown in Fig. 2, a single radio peak with good chromatographic characteristics was obtained: its yield was very high, and the reproducibility was good. The methylation was proved to be almost quantitative, as generally accepted, and the adequacy of the method was verified.

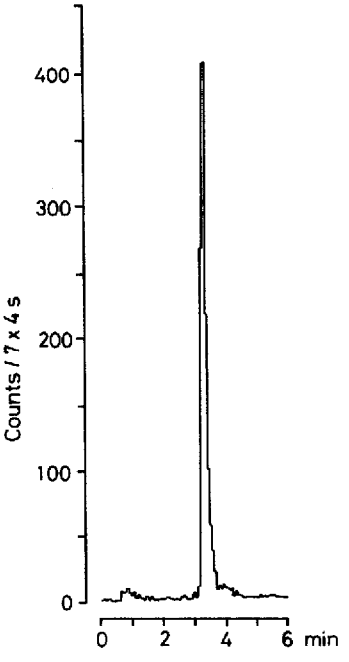

Fig. 2. Radiochromatogram of [^{3}H]arachidonic acid treated with CH_2N_2. Injected radioactivity, 120 Bq. GC peak yield (mean ± S.D., $n = 5$) = 91.1 ± 2.72%.

6-Keto-$PGF_{1\alpha}$ has attracted special interest recently, in that it can reflect the concentration of PGI_2 in biological fluids. No paper has yet appeared concerning the close examination of its optimal derivatization conditions for GC. The substance has three kinds of site to be derivatized, *viz.* carboxyl, carbonyl and hydroxyl moieties. The sites are usually derivatized into methyl ester (ME) with CH_2N_2, methyloxime (MO) with MOX, and silyl ether with various silylation reagents, respectively.

[^{3}H]6-Keto-$PGF_{1\alpha}$ was injected into the capillary GC–SARD system after derivatization under the usual conditions [7,10]. A Sephadex LH-20 column was used to eliminate derivatization reagents. The resulting radiochromatograms are shown in Fig. 3. With any silylation reagents the background was significantly increased. This was considered to arise from side-reactions during derivatization

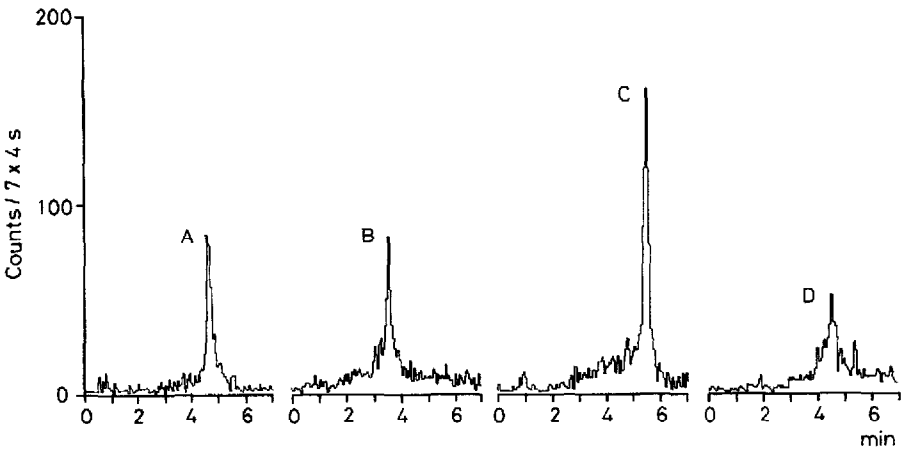

Fig. 3. Radiochromatograms of [^{3}H]6-keto-PGF$_{1\alpha}$-ME-MO treated with various silylation reagents: (A) ME-MO-TMS derivative; (B) ME-MO-tBDMS derivative; (C) ME-MO-DMiPS derivative; (D) ME-MO-DMnPS derivative. GC peak yield (average and S.D. of three samples derivatized separately): (A) 25.8 ± 6.97%; (B) 22.3 ± 5.72%; (C) 39.8 ± 4.38%; (D) 19.4 ± 5.11%.

and thermal decomposition during GC processes. GC peak yields proved to be relatively low (20–40%) and varied considerably with different silylation reagents. Other analytical methods cannot determine the GC peak yields, and do not detect the side-reactions or the thermal decomposition at all because it is impossible to discriminate between the substances under consideration and the derivatization reagents. The recovery yields from the Sephadex LH-20 column were 80–90% in all cases. The reproducibility of GC peak yields was extremely low in all experiments. The low GC peak yields with poor reproducibility were ascribed to the silylation rather than the methoximation step, since the substance has three silylation sites. The highest GC peak yield and precision were obtained with DMiPSI. DMiPSI was developed by Miyazaki *et al.* [7], and has been accepted to be one of the most effective silylation reagents for GC–MS analyses of PGs, because of its good GC and MS characteristics and quantitative reaction. The derivatization has usually been performed at room temperature for 60 min. However, these conditions were shown to be inadequate from the above experimental results. The optimal silylation conditions with DMiPSI were investigated by monitoring the GC peak yield.

[^{3}H]6-Keto-PGF$_{1\alpha}$ was injected after treatment with CH_2N_2, MOX, and DMiPSI at various temperatures and for different reaction times. The results are shown in Fig. 4. The GC peak yields were increased up to 70–80% by carrying out the reaction at less than 8°C for the appropriate time. A typical radiochromatogram is shown in Fig. 5, in which silylation was performed at 8°C for 45 min. The conditions gave only a slight elevation of the background, the peaks were of good shape, and the reproducibility was significantly improved.

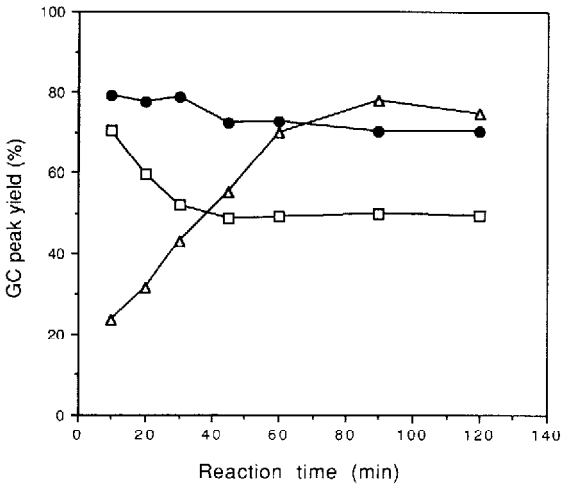

Fig. 4. Effect of the reaction time on GC peak yield of 6-keto-$PGF_{1\alpha}$-ME-MO-DMiPS, at three temperatures. Each point represents the mean for three samples derivatized separately. (□) 25°C; (●) 8°C; (△) −5°C.

In conclusion, it has been shown that ^3H-labelled substances can be analysed precisely by using GC–SARD over a wide range of experimental conditions, and that the GC peak yields can be easily determined by the use of [^{3}H]hexadecane or [^{3}H]androstenedione as a standard substance. The usefulness of determining the GC peak yields was demonstrated by optimizing the derivatization conditions of 6-keto-$PGF_{1\alpha}$. This method is considered to be more effective for the investigation of the GC analytical conditions for samples contaminated with biological components.

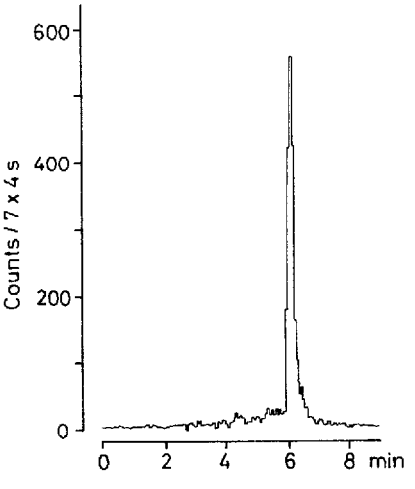

Fig. 5. Radiochromatogram of [^{3}H]6-keto-$PGF_{1\alpha}$-ME-MO silylated with DMiPSI under the optimal conditions (8°C for 45 min). GC peak yield (average and S.D. of five samples derivatized separately) = 72.3 ± 3.27%.

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