Application of Gas Chromatograph-Mass Spectrometer-Computer System to Evaluation of ^{14}C -Labelled Compounds

Hiroshi KANAMARU, Iwao NAKATSUKA and Akira YOSHITAKE

Takarazuka Research Center, Sumitomo Chemical Co., Ltd.

2-1, Takatsukasa 4-chome, Takarazuka-shi, Hyogo Pref. 665

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Usefulness of gas chromatograph-mass spectrometer-computer system (GC-MS-CPU) not only for measurement of specific activities of ^{14}C -labelled compounds in a mixture but also for evaluation of ^{14}C -labelled compounds in terms of examining their purities and elucidating chemical structures of the impurities was proved.

A sample of methyl 2-(*p*-chlorophenyl- $^{14}\text{C}_n$)-3-methylbutyrate (III) synthesized from *p*-chlorophenyl- $^{14}\text{C}_n$ -acetonitrile (VI) was analyzed by GC-MS-CPU, and it was found that the labelled compound was contaminated with a small amount of the corresponding *m*-isomer (IV) having a very high specific activity. Further examination suggested that the contaminating *m*-isomer (IV) originated from *m*-chlorophenyl- $^{14}\text{C}_n$ -acetonitrile (IX) which had already contaminated in the starting material (VI), and also that cyanomethylation of *p*-dichlorobenzene- $^{14}\text{C}_n$ (VIII) by benzene-type reaction resulted in producing a mixture of *p*- and *m*-chlorophenyl- $^{14}\text{C}_n$ -acetonitriles (VI, IX).

Key Words: gas chromatograph-mass spectrometer-computer system, specific activity measurement, structure elucidation, 2-(*p*-chlorophenyl- $^{14}\text{C}_n$)-3-methylbutyric acid

1. Introduction

In the preceding paper¹⁾, we described a method for measuring specific activities of ^{14}C -labelled compounds by gas chromatograph-mass spectrometer-computer system (GC-MS-CPU), by which specific activities can be determined with practical accuracy, precision, and convenience. It was also suggested that the method was additionally remarkable merits such as providing information on the purity and structure of the proposed ^{14}C -labelled compounds and possibly on the structure of impurities. As described in the preceding paper¹⁾, the principle for determination of the specific activity of a ^{14}C -labelled compound by GC-MS-CPU is quite different from the conventional method of measuring specific activity from the radioactivity of a known weight of a labelled compound. This method needs only good mass spectra of both of a ^{14}C -labelled compound and the corresponding unlabelled sample. Even though the labelled compound is in a mixture,

the mass spectra can be measured without any isolation of the labelled compound by the GC-MS part of this system, as long as the labelled compound is separated on the GC column employed. Then the mass spectra are analyzed by the CPU of the system to provide the specific activity of the labelled compound.

In the present paper, we describe an application of GC-MS-CPU to evaluate ^{14}C -labelled

Fig. 1 Structures of 2-(*p*-chlorophenyl- $^{14}\text{C}_n$)-3-methylbutyric acid (I) and related compounds.

compounds in terms of not only determination of the specific activity and purity but also structure elucidation of impurities; as a representative example, a successful application to solve some problems occurred in the synthesis of 2-(*p*-chlorophenyl- $^{14}\text{C}_n$)-3-methyl-butyric acid (CPBA- $^{14}\text{C}_n$) (I) being discussed. The labelled compound was prepared as an important intermediate to synthesize fenvalerate-(*p*-chlorophenyl- $^{14}\text{C}_n$) (V)²⁾ for use in metabolic studies of fenvalerate which is one of the most important and widely used pesticides in the world (Fig. 1).

2. Experimental

2.1 Apparatuses and methods

Measurement of mass spectra and calculation of specific activity was carried out with a LKB-9000 gas chromatograph-mass spectrometer combined with a PAC-300 DGB computed data system (Shimadzu, Kyoto, Japan). The principle and method for the specific activity measurement by GC-MS-CPU were described in detail in the preceding paper¹⁾. The measuring condition for GC-MS-CPU analysis of methyl 2-(*p*/*m*-chlorophenyl- $^{14}\text{C}_n$)-3-methylbutyrate (III, IV) was as follows; 1) GC-condition: column Apieson L (2%) on Chromosorb W (2 m $\times$ 3 mm ID, glass column), column temperature 100 to 200°C (raising rate 4°C/min), carrier gas He (flow rate 30 ml/min), injection temperature 250°C, separator temperature 220°C; 2) MS-condition: electron energy 70 eV, chamber temperature 250°C, trap current 60 μA , acceleration potential 3.5 kV, slit 0.20/0.15 mm, scanning speed 8, gain 5; 3) mass chromatographic condition: interval 5.5 s, off-set time 14 min, stop time 17 min, mass range 10–300 (*m/z*).

Radio-gas chromatography was carried out with a Yanaco G-80 gas chromatograph combined with a RD-4 radiodetector (Yanagimoto Seisakusho, Kyoto, Japan). The analytical condition was as follows: column Apieson L (2%) on Chromosorb W (3 m $\times$ 3 mm ID, glass column), column temperature 130°C, carrier gas He (30 ml/min), counting gas propane (50 ml/min).

Radio-thin layer chromatography (TLC) was measured on Merck TLC plate Silica Gel

60 F₂₅₄ (Kanto Chemical, Tokyo, Japan) with an Aloka TLC-101U scanner (Aloka, Tokyo, Japan). Radioactivity was measured with a Packard Tri-Carb Model 3385 liquid scintillation counter (Amco, Tokyo, Japan). Nuclear magnetic resonance (NMR) spectra were measured with a Hitachi R-40 (90 MHz) NMR spectrometer (Hitachi, Tokyo, Japan).

2.2 2-(*p*/*m*-Chlorophenyl- $^{14}\text{C}_n$)-3-methylbutyronitriles (VII/X)

A mixture of *p*/*m*-chlorophenyl- $^{14}\text{C}_n$ -acetonitriles (VII/X) (1.12 g, 7.41 mmol, 581 MBq/mmol, 15.7 mCi/mmol) purchased from a manufacturer, isopropyl bromide (1.40 g, 11.4 mmol) and benzyl triethylammonium chloride (0.04 g, 0.19 mmol) in 44% sodium hydroxide solution (2.5 ml) was heated under stirring at 55°C for 3 h and to the mixture was added benzyl triethylammonium chloride (0.05 g, 0.20 mmol). Then the mixture was heated at the same temperature for further 3 h. After addition of water (30 ml), the mixture was extracted with ether. The extract was washed with water, dried over sodium sulfate and evaporated to dryness. The residue was chromatographed on silica gel and eluted with hexane-ether (97/3 v/v) to give 2-(*p*/*m*-chlorophenyl- $^{14}\text{C}_n$)-3-methylbutyronitriles (VII/X) (1.04 g, 581 MBq/mmol, 15.7 mCi/mmol); the radiochemical purity 98% on TLC (hexane/ether=5/1, *R_f* value=0.29) and the radiochemical purity 99% on GC (2% Silicone OV-17 on Chromosorb W, 1.5 m $\times$ 3 mm ID glass column, column temperature 130°C, carrier gas He 32 ml/min, retention time 6.4 min).

2.3 2-(*p*/*m*-Chlorophenyl- $^{14}\text{C}_n$)-3-methylbutyric acids (*p*/*m*-CPBA- $^{14}\text{C}_n$) (I/II)

A mixture of 2-(*p*/*m*-chlorophenyl- $^{14}\text{C}_n$)-3-methylbutyronitriles (VII/X) (3 160 MBq, 85.5 mCi, 1.04 g, 5.45 mmol) and 64% sulfuric acid (2.5 ml) was refluxed for 7 h. After cooling, water (10 ml) was added to the reaction mixture and the mixture was extracted with ether. The ethereal solution was extracted with 5% sodium hydroxide solution (65 ml) and the aqueous solution was acidified with hydrochloric acid. Re-extraction of the aqueous mixture with ether followed by evaporation of the ex-

tract gave 2-(*p*/*m*-chlorophenyl- $^{14}\text{C}_n$)-3-methylbutyric acids (I/II) (946 mg, 4.45 mmol, 581 MBq/mmol, 15.7 mCi/mmol); the radiochemical purity 98% on TLC (chloroform/methanol=3/2, R_f value=0.51).

2.4 Methyl 2-(*p*/*m*-chlorophenyl- $^{14}\text{C}_n$)-3-methylbutyrates (III/IV)

2-(*p*/*m*-Chlorophenyl- $^{14}\text{C}_n$)-3-methylbutyric acids (I/II) (168 MBq, 4.55 mCi, 61.6 mg, 0.29 mmol) was allowed to react with an excess of diazomethane (1.50 mmol) in ether (5 ml) at room temperature for 3 h. The reaction mixture was diluted with ether, washed with 5% sodium bicarbonate solution and water successively, and then dried over anhydrous sodium sulfate. Evaporation of the ethereal solution gave methyl 2-(*p*/*m*-chlorophenyl- $^{14}\text{C}_n$)-3-methylbutyrates (III/IV) in a quantitative yield; IR ($\nu_{\max}$): 1735 cm^{-1} (CO).

2.5 *p*/*m*-Chlorophenylacetonitriles (Kame-tani's method)

To a stirring solution of metallic sodium (1.44 g, 62.6 mg-atom) in liquid ammonia (30 ml) was added ferric chloride (10 mg, 0.062 mmol) under cooling in a dry ice-acetone bath at -78°C , and the mixture was stirred at the same temperature for 3 h. To the mixture was added acetonitrile (1.32 g, 32.2 mmol) within 5 min, followed by rapid addition of *p*-dichlorobenzene (1.51 g, 0.4 mmol). After the mixture was stirred at -78°C for 4 h, the excess of sodium amide was decomposed by addition of ammonium chloride (3.20 g, 59.8 mmol), and then ammonia was vaporized at room temperature. After addition of water (10 ml) and ethanol (5 ml), the resultant mixture was filtered with celite, and the filtrate was extracted with benzene. The extract was washed with 10% hydrochloric acid and water, dried over anhydrous sodium sulfate. Evaporation of the extract gave an oily product of *p*/*m*-chlorophenylacetonitriles. The product was analyzed by gas chromatography (15% Siponate DS-10 on Uniport B, 5 m $\times$ 3 mm ID glass column, column temperature 165°C , carrier gas He 40 ml/min, retention times *o*- 13.3 min, *m*- 21.8 min, *p*- 24.4 min).

2.6 Unlabelled authentic samples

2-(*p*-Chlorophenyl)-3-methylbutyric acid and its methyl ester, the unlabelled authentic samples of I and III, were prepared by the same procedure described above from *p*-chlorophenylacetonitrile, which was purchased from Aldrich Chemical Co. (Wisconsin, U.S.A.). 2-(*m*-Chlorophenyl)-3-methylbutyric acid and its methyl ester, the unlabelled authentic samples of II and IV, were synthesized in the similar manner from *m*-chlorophenylacetonitrile purchased from Aldrich Chemical Co.

Their structures were confirmed by NMR, IR and MS as follows. 2-(*p*-Chlorophenyl)-3-methylbutyric acid: NMR (δ , ppm; CDCl_3) 0.67 (3H, d, $J=6$ Hz, CH_3), 1.03 (3H, d, $J=6$ Hz, CH_3), 2.08–2.47 (1H, m, $-\text{CH}(\text{CH}_3)_2$), 3.08 (1H, d, $J=10$ Hz, methyne H), 7.23 (4H, s, aromatic H), 11.06 (1H, broad s, $-\text{COOH}$); IR ($\nu_{\max}$, cm^{-1} ; nujol) 1705 (CO); MS (m/z) 212/214 (M^+), 170/172 (base peak), 152/154. Methyl 2-(*p*-chlorophenyl)-3-methylbutyrate: IR ($\nu_{\max}$, cm^{-1} ; liquid film) 1735 (CO); MS (m/z) 226/228 (M^+), 184/186 (base peak), 167/169, 152/154, 125/127. 2-(*m*-Chlorophenyl)-3-methylbutyric acid: NMR (δ , ppm, CDCl_3) 0.70 (3H, d, $J=6$ Hz, CH_3), 1.05 (3H, d, $J=6$ Hz, CH_3), 2.05–2.49 (1H, m, $-\text{CH}(\text{CH}_3)_2$), 3.08 (1H, d, $J=10$ Hz, methyne H), 7.00–7.40 (4H, m, aromatic H), 10.17 (1H, broad s, $-\text{COOH}$); IR ($\nu_{\max}$, cm^{-1} ; nujol) 1705 (CO); MS (m/z) 212/214 (M^+), 170/172 (base peak), 152/154. Methyl 2-(*m*-chlorophenyl)-3-methylbutyrate: IR ($\nu_{\max}$, cm^{-1} ; liquid film) 1735 (CO); MS (m/z) 226/228 (M^+), 184/186 (base peak), 167/169, 152/154, 125/127.

3. Results and Discussion

The procedure²⁾ for the synthesis of tentatively so-called 2-(*p*-chlorophenyl- $^{14}\text{C}_n$)-3-methylbutyric acid (I) are illustrated in Fig. 2. The starting material was tentatively so-called *p*-chlorophenyl- $^{14}\text{C}_n$ -acetonitrile (VI) which was supplied as a custom preparation by a manufacturer. At first, the ^{14}C -labelled starting material was diluted with unlabelled authentic *p*-chlorophenylacetonitrile to give tentatively so-called *p*-chlorophenyl- $^{14}\text{C}_n$ -acetonitrile (VI) having a specific activity of 581 MBq/mmol (15.7 mCi/

Fig. 2 Scheme for the syntheses of 2-(*p*-chlorophenyl- $^{14}\text{C}_n$)-3-methylbutyric acid (I) and *p*-chlorophenyl- $^{14}\text{C}_n$ -acetonitrile (VI).

mmol). Condensation of VI with isopropyl bromide in the presence of benzyl triethylammonium chloride in an alkaline solution gave a crude product. The crude product was purified by column chromatography on silica gel to give tentatively so-called 2-(*p*-chlorophenyl- $^{14}\text{C}_n$)-3-methylbutyronitrile (VII) with 99% radiochemical purity on TLC and GC analysis, having the specific activity of 581 MBq/mmol. Hydrolysis of VII with 64% sulfuric acid followed by purification with 5% sodium hydroxide solution and hydrochloric acid yielded tentatively so-called *p*-CPBA- ^{14}C (I) in 60% yield from VII.

TLC analysis of the suspected *p*-CPBA- $^{14}\text{C}_n$ (I) thus obtained revealed that its purity seemed to be radiochemically and chemically over 98%, and determination of the specific activity in a usual manner proved that I had the same specific activity (581 MBq/mM) as that of VI and VII. From these facts, the labelled compound was considered to be pure enough for the following reactions. However, examining its NMR spectrum in detail, it was found that the signals at δ 7.20–7.40 of the aromatic proton region appeared in rather splitted pattern as compared with those in the NMR spectrum of unlabelled authentic sample. The fact did suggest a possibility that tentatively so-called *p*-CPBA- $^{14}\text{C}_n$ (I) was contaminated with a small amount of an unknown impurity which could not be detected by the TLC analysis mentioned above.

To confirm the purity of the *p*-CPBA- $^{14}\text{C}_n$ (I) in more detail, therefore, it was converted to the corresponding methyl ester which was expected to be effectively analyzed by radio-gas chromatography. The esterification was achieved by treatment of I with diazomethane, giving a

Fig. 3 Radio-gas chromatogram of methyl 2-(*p*/*m*-chlorophenyl- $^{14}\text{C}_n$)-3-methylbutyrate (III/IV).

neutral product in a quantitative yield. The product showed a single spot on TLC with some different solvent systems, and showed an infrared absorption at 1735 cm^{-1} for the ester carbonyl. Radio-gas chromatography of the product on 2% Apiezon L glass column revealed that it was a mixture of two compounds with different specific activities whose retention times were 18.4 and 16.2 min (Fig. 3). The percent ratio of the compound having the retention time of 18.4 min in the mixture was chemically 89% but radiochemically 44% when measured the areas under the graphs by integrators.

To explore the structure and the specific activity of each compound, the mixture was subjected to the analysis by GC-MS-CPU, by which the structure and the specific activity can be expected to be determined without any isolation of each compound. The mass spectrum of the ^{14}C -labelled compound with the GC-retention time of 18.4 min gave the molecular ion cluster at m/z 226 (Fig. 4(a)), indicating that the unlabelled molecule of the ^{14}C -labelled compound had a composition of $\text{C}_{12}\text{H}_{10}\text{O}_2\text{Cl}$ and the ^{14}C -labelled compound was elucidated to be methyl 2-(*p*-chlorophenyl- $^{14}\text{C}_n$)-3-methylbutyrate (III). This was easily confirmed by the mass spectrum in comparison with that of the unlabelled authentic sample of III. The findings that the ^{14}C -labelled compound having the retention time of 16.2 min showed the molecular ion cluster at m/z 226 in its mass spectrum

Fig. 4 Mass spectra of methyl 2-(*p*-chlorophenyl- $^{14}\text{C}_n$)-3-methylbutyrate (III) (a) and methyl 2-(*m*-chlorophenyl- $^{14}\text{C}_n$)-3-methylbutyrate (IV) (b).

(Fig. 4(b)) suggested that this compound was an isomer with the same composition as III and with a different aromatic substitution of chlorine. This was supported by detailed comparison of the mass spectra of both ^{14}C -labelled compounds, in which some differences were found in relative peak intensities of two fragment ion peaks at m/z 152 and at m/z 125 although their fragmentation patterns were similar to each other. A confirmation of the structural conclusion that this ^{14}C -labelled compound is methyl 2-(*m*-chlorophenyl- $^{14}\text{C}_n$)-3-methylbutyrate (IV) was found in its mass spectrum which

showed the identical fragmentation pattern and peak intensities with those of the unlabelled authentic sample of IV as long as unlabelled molecule concerned. Furthermore, the retention times of the ^{14}C -labelled esters on GC were identical with those of the unlabelled authentic samples.

Table 1 shows specific activities of the ^{14}C -labelled esters (III and IV) measured by GC-MS-CPU. *p*-Me-CPBA- $^{14}\text{C}_n$ (III) was found to have a relatively low specific activity of 285 MBq/mmol (7.69 mCi/mmol) whereas there was found an extensively high specific activity of 3 064 MBq/mmol (82.8 mCi/mmol) for *m*-Me-CPBA- $^{14}\text{C}_n$ (IV). Radiochemical purity of *p*-Me-CPBA- $^{14}\text{C}_n$ (III) in the mixture was calculated to be 45% from its specific activity (285 MBq/mmol, 7.69 mCi/mmol) and chemical purity (90%) measured by the total ion monitor of GC-MS-CPU; being coincident with that obtained by the radio-gas chromatography mentioned above.

On the basis of all of these results, it could be concluded that tentatively so-called *p*-CPBA- $^{14}\text{C}_n$ (I) presently prepared by the series of reactions was contaminated with 2-(*m*-chlorophenyl- $^{14}\text{C}_n$)-3-methylbutyric acid (II) in the extent of chemically about 10% and radiochemically about 55%. This *m*-isomer (II) having such a high specific activity as 3 064 MBq/mmol (82.8 mCi/mmol) was assumed to originate from a by-product which might be yielded by a certain reaction of a very early step in the synthetic procedure.

Information from the manufacturer disclosed

Table 1 Measurement of specific activities of methyl 2-(*p*-chlorophenyl- $^{14}\text{C}_n$)-3-methylbutyrate (III) and methyl 2-(*m*-chlorophenyl- $^{14}\text{C}_n$)-3-methylbutyrate (IV) by GC-MS-CPU

Compound	Relative abundance of $^{14}\text{C}_n$ -labelled molecule (x_n)*						Specific activity**
	x_0	x_1	x_2	x_3	x_4	x_5	
<i>p</i> -Isomer (III)	100	4.5	2.6	0.6	0.4	—	285 (7.69)
<i>m</i> -Isomer (IV)	100	118.5	89.8	40.7	12.4	2.2	3064 (82.8)

* Molecular ions were used for measurement.

$$x_n = a_n - \sum_{k=1}^n \left(\frac{b_k}{b_0} x_{n-k} \right)$$

where x_n =the relative intensity of the peak originating from $^{14}\text{C}_n$ -labelled molecule
 a_n =the relative intensity of the peak originating from $^{14}\text{C}_n$ -labelled compound
 b_n =the relative intensity of the peak originating from unlabelled standard
 $x_0 = a_0 = b_0 = 100$ (normalization)

** Specific activity = $2.31 \times (\sum n x_n / \sum x_n)$ GBq/mmol; $62.4 \times (\sum n x_n / \sum x_n)$ mCi/mmol.

that tentatively so-called *p*-chlorophenyl- $^{14}\text{C}_n$ -acetonitrile (IV) which we used as the starting material had been synthesized from *p*-dichlorobenzene- $^{14}\text{C}_n$ (VIII) by applying the method of Kametani, et al.³⁾ (Fig. 2). Kametani, et al. reported that in the case of *p*-dichlorobenzene when reacted with acetonitrile in the presence of sodium amide, the cyanomethylation occurred at the position attached by chlorine to give only *p*-chlorophenylacetonitrile but not *m*-isomer. However, suppose that this reaction might go through a benzyne intermediate, there would be a possibility of the benzyne being attacked by acetonitrile at both sites of *p*- and *m*-position to yield a mixture of *p*- and *m*-chlorophenylacetonitrile. Confirmation of this assumption was achieved by re-examining this reaction with the use of the same reaction condition as described by Kametani, et al.³⁾ Results shown in Table 2 evidently indicate that *p*-dichlorobenzene under this condition gives a mixture of *p*- and *m*-chlorophenylacetonitrile in a ratio of approximately 1/1. This observation indicates that *m*-CPBA- $^{14}\text{C}_n$ (II) detected by us originated most probably from *m*-chlorophenyl- $^{14}\text{C}_n$ -acetonitrile (IX) which had been produced from *p*-dichlorobenzene- $^{14}\text{C}_n$ (VIII) during cyanomethylation of benzyne-type reaction and contaminated in the starting material (VI) without having been detected by the analytical method employed. Furthermore, the manufacturer informed the fact that the cyanomethylation was

Table 2 Ratios of *p*/*m*-chlorophenylacetonitrile produced by benzyne reaction

Run	<i>p</i> -	/	<i>m</i> -
1	44	/	56
2	55	/	45
3	51	/	49

performed by using *p*-dichlorobenzene- $^{14}\text{C}_n$ (VIII) having a very high specific activity over 2 960 MBq/mmol (80 mCi/mmol) and then the product was diluted with unlabelled authentic *p*-chlorophenylacetonitrile. The fact well explains the contamination of *m*-CPBA- $^{14}\text{C}_n$ (II) having the high specific activity (3 064 MBq/mmol) in *p*-CPBA- $^{14}\text{C}_n$ (I) with the relatively low specific activity (285 MBq/mmol) and the chemical ratio of these compounds as well.

In conclusion, the GC-MS-CPU was proved to be practically useful not only for determination of specific activities of ^{14}C -labelled compounds in a mixture but also for evaluation of them in terms of examining purities and elucidating chemical structures of impurities without any isolation.

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要 旨

 ^{14}C -標識化合物の品質評価へのガスクロマトグラフ-
マススペクトロメータ-コンピュータシステムの適用

金丸 博, 中塚 巖, 吉武 彬

住友化学工業(株)宝塚総合研究所

665 兵庫県宝塚市高司 4-2-1

ガスクロマトグラフ-マススペクトロメータ-コンピュータシステム(GC-MS-CPU)が混合物状態の ^{14}C -標識化合物の比放射能測定だけでなく、純度検定や不純物の構造解明等、 ^{14}C -標識化合物の品質評価にも有用であることを明らかにした。

p -クロロフェニル- $^{14}\text{C}_n$ -アセトニトリル(VI)より合成した 2-(p -クロロフェニル- $^{14}\text{C}_n$)-3-メチル酪酸メチル(III)を GC-MS-CPU 分析したところ、非常に高い比放射能を有する m -異性体(IV)が少量混入していた。そこでさらに詳細な検討を行ったところ、この m -異性体(IV)は、出発物質(VI)にすでに混入していた m -クロロフェニル- $^{14}\text{C}_n$ -アセトニトリル(V)に由来するものであること、および p -ジクロロベンゼン- $^{14}\text{C}_n$ (VII)のベンザイン反応によるシアノメチル化によって p, m -クロロフェニル- $^{14}\text{C}_n$ -アセトニトリル混合物(VI, IX)が生成したことが推定された。