High Sensitivity Analysis of Indirubin by Silylation Using GC/MS

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After adding the silylating agents N, O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) or N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) to the indirubin solution and leaving for 1 hr at 60°C, the color remained red. Indirubin could be measured by GC/MS after replacing the active hydrogen on the amino group with $-Si(CH_3)_3$ or $-Si(CH_3)_2C_2H_5$ groups. However, peak tailing was observed and the quantitative and detection limits were not sensitive enough for practical use. Indirubin silylated at four sites was observed under the reaction conditions as follows; solvent dichloromethane: acetone (8 : 2), 90°C reaction temperature, 1 hr reaction time, BSTFA derivative, pyridine catalyst. The color of the solution changed from red to colorless. Retention time appeared to be faster and the peak shape improved. Under these conditions, the quantitative and detection limits of indirubin were 5 ppb and 0.1 ppb, respectively.

Key words —— indirubin, silylation, GC/MS, N, O-bis(trimethylsilyl) trifluoroacetamide, derivative

INTRODUCTION

Numerous estrogenic chemicals that are found in the environment cause abnormalities in growth and reproduction.[1–8] Adachi *et al*. recently reported that indirubin was present at an average concentration 0.2 nM in the urine of normal donors, and that it showed extremely strong aryl hydrocarbon receptor (AhR) ligand activity that was two orders of magnitude stronger than that for 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD).[9] We therefore believe that an analytical method capable of detecting indirubin in the order of several ppb or lower is needed to evaluate risks in living organisms. We have been developed analytical methods of environmental estrogens and evaluation methods of biological activities.[10–17] However, our preliminary experiments with HPLC/MS indicated that quantitative and detection limits of indirubin were 500 ppb and 200 ppb, respectively. These values are insufficient for practical analysis. Establishing a new and sensitive method for analyzing lower concentrations is urgently necessary. Therefore, we attempted to develop a highly sensitive analytical method for indirubin using derivatization of indirubin in order to enhance GC/MS.

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MATERIALS AND METHODS

Reagents —— N, O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) (SPELCO Ltd., U.S.A.) were used as chemical reagents for silylation. Other reagents were of analytical-reagent grade for analyzing pesticide residues (Kanto Chemical, Japan) unless otherwise stated. Indirubin was kindly provided by Dr. Miyairi (College of Pharmacy, Nihon University, Chiba, Japan). Indirubin solution of 10 ppm dissolved in 1.0 ml dichloromethane and 200 μl derivative reagent including a catalyst were mixed in a 2.0-ml glass vial. The vial was sealed, and left to stand for 1 hr at the specified temperatures (R. T. to 130°C) in a drying

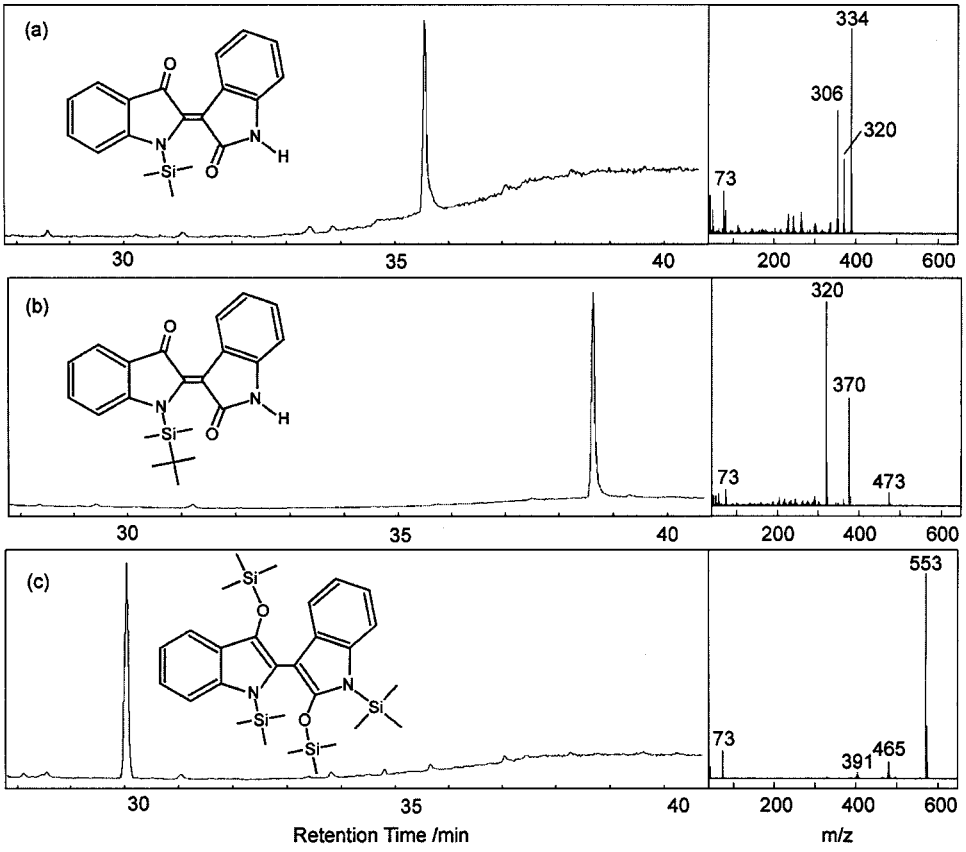

Fig. 1. Chromatographs, Structural Formulas and Mass Spectra of Indirubin after Reaction for 1 hr at 60°C
(a) BSTFA as silylating reagent, (b) MTBSTFA as silylating reagent, and (c) BSTFA as silylating reagent with pyridine as catalyst.

oven. The reaction solution was then analyzed by GC/MS.

GC/MS System —— Analysis was conducted on a GC (CP-3800, Varian, U.S.A.) column equipped with an ion trap MS detector (Saturn 2000, Varian) and a capillary column (DB-5ms; length, 30 m; film thickness, 0.25 mm; diameter, 0.25 mm; J & W Scientific, U.S.A.), in splitless mode (10 psi, split ratio = 25) using helium as the carrier gas. Injection volume was 1 μl. The split vent was opened 1 min after starting the analysis. Injection port and ion trap temperatures were set at 300 and 250°C, respectively. Column temperature was initially maintained at 35°C for 1 min and was then increased to 250°C at a rate of 10°C/min. After the column temperature was at 250°C for 10 min, it was increased again to 300°C at a rate of 10°C/min and maintained at that level for another 5 min. MS analysis was carried out using EI auto mode with an MS scan speed of 1.2 scan/sec and an MS detector voltage of 1450 V.

RESULTS AND DISCUSSION

Indirubin has three functional groups (an amido, an amino, and a carbonyl group), and a molecular weight of 262. At a concentration of several ppm, the solution is colored violet. The indirubin peak without silylation did not appear on the chromatogram, because the compound has a relatively high polarity and molecular weight. After adding the silylating agents BSTFA or MTBSTFA to the indirubin solution and leaving for 1 hr at 60°C, the color remained unchanged. Total ion chromatographs of the solutions are shown in Figs. 1(a) and (b). The mass spectra are shown on the right. Indirubin could be measured by GC/MS after replacing the active hydrogen on the amino group with $-Si(CH_3)_3$ or $-Si(CH_3)_2C_2H_5$ groups. However, peak tailing was observed on each chromatograph. It appears that the quantitative and detection limits are 10 ppm and 5 ppm, respectively, and are not sensitive enough for practical use. We believe that the primary cause of this phenomenon is the remaining amino and carbonyl groups, which cause a slight polarity. There-

fore, when analyzing low concentrations, the molecule is adsorbed to the active site on the column and cannot reach the mass detector.

In the next step, we attempted to depolarize indirubin in order to detect lower concentrations by adding catalysts and changing the reaction conditions. As a result, indirubin silylated at four sites was observed and is shown in Fig. 1(c). The best reaction conditions were as follows; solvent dichloromethane:acetone (8 : 2), 90°C reaction temperature, 1 hr reaction time, BSTFA derivative (200 μl), pyridine catalyst. The color of the solution changed from red to colorless or weak yellow. Addition of chlorotrimethylsilane as a catalyst resulted in lower yields. MTBSTFA was not able to silylate indirubin at the four sites under any conditions. A tarry compound was produced in the solution at temperatures above 100°C, and we did not observe the silylated indirubin peak, despite silica-gel column cleanup. There were no peaks for indirubin that was silylated at two or three sites on the chromatographs. Retention time appeared to be faster and the peak shape improved, as shown in Fig. 1(c). Under these conditions, the quantitative and detection limits of indirubin were 5 ppb and 0.1 ppb, respectively. These values seem to be sufficiently sensitive for practical analysis of samples.

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