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# Measurement of glycidol hemoglobin adducts in humans who ingest edible oil containing small amounts of glycidol fatty acid esters

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ABSTRACT

Hemoglobin (Hb) adducts are frequently used to address and/or monitor exposure to reactive chemicals. Glycidol (G), a known animal carcinogen, has been reported to form Hb adducts. Here, we measure G adduct levels in humans who daily ingest DAG oil, an edible oil consisting mainly of diacylglycerol. Since DAG oil contains a small amount of glycidol fatty acid esters (GEs), possible exposure to G released from GEs has been raised as a possible concern. For measurement of Hb adducts, we employed the N-alkyl Edman method reported by Landin et al. (1996) using gas chromatography–tandem mass spectrometry with minor modifications to detect G-Hb adducts as N-(2,3-dihydroxy-propyl)valine (diHOPrVal). Blood samples were collected from 7 DAG oil users and 6 non-users, and then G-Hb adduct levels were measured. G-Hb adducts were detected in all samples. The average level of diHOPrVal was 3.5 ± 1.9 pmol/g globin in the DAG oil users and 7.1 ± 3.1 pmol/g globin in the non-users. We conclude that there is no increased exposure to G in individuals who daily ingest DAG oil.

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## 1. Introduction

Diacylglycerol (DAG) oil is a unique edible oil that is defined to contain DAG at a concentration of 80% (w/w) or greater. DAG oil has been shown to have a preventive effect on body fat accumulation that had been marketed by Kao Corporation as Food for Specified Health Uses (FOSHU) in Japan (Nagao et al., 2000; Flickinger and Matsuo, 2003; Nishide et al., 2004; Yasunaga et al., 2004).

In March 2009, the German Federal Institute for Risk Assessment (BfR) expressed safety concerns regarding glycidol fatty acid esters (GEs) present in refined edible oils due to possible release of a known animal carcinogen, glycidol (G), from GEs during digestion in humans (BfR, 2009a,b; Bakhiya et al., 2011). GEs occur as a by-product of the general process of deodorization during fat and oil production.

In June 2009, DAG oil was found to contain small amounts of GEs that were nonetheless at levels considerably higher than other commercial edible oils. According to a recent report by Masukawa et al. (2010), DAG oil contains GEs (summed value of each GE, i.e. C16:0-GE, C18:0-GE, C18:1-GE, C18:2-GE, and C18:3-GE) at a concentration of 269 μg/g, whereas two commercial edible oils that mainly consisted of triacylglycerol contained GEs at significantly lower concentrations of 22.8 μg/g and 6.7 μg/g, respectively. As a result, the company temporarily halted sales of DAG oil and related products until GE levels could be reduced, and the FOSHU status of DAG oil was voluntarily revoked although no alleged safety issues were confirmed. As BfR stated, there was no suitable analytical method to analyze GE and G levels under conditions of actual exposure, and little information is available to address the conversion of GEs into G in the human body.

Hemoglobin (Hb) adducts have been used to address and/or monitor exposure to various reactive chemicals (Törnqvist and Landin, 1995; Boogaard, 2002; Ogawa et al., 2006). Compared with other classical biomarkers, such as target chemicals and their metabolites in blood and urine and modified enzymes, Hb adducts have advantages in terms of their abundance, accessibility of methods for chemical identification, and well-defined life spans in blood. Based on the average life span of a red blood cell in humans, Hb adducts can indicate exposure levels to target chemicals over the previous 120 days (Shemin and Rittenberg, 1946; Osterman-Golkar et al., 1976).

*Abbreviations:* BDHQ, brief-type self-administered diet history questionnaire; DAG, diacylglycerol; G, glycidol; GE, glycidol fatty acid esters; diHOPrVal, N-(2,3-dihydroxy-propyl)valine; Hb, hemoglobin; GC, gas chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PFPTH, pentafluorophenylthiohydantoin; PFPITC, pentafluorophenyl isothiocyanate; NCI, negative ion chemical ionization; FOSHU, Food for Specified Health Uses; BfR, German Federal Institute for Risk Assessment; LC, liquid chromatography; NMR, nuclear magnetic resonance.

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G has been reported to form Hb adducts. Landin et al. demonstrated sensitive detection of N-(2,3-dihydroxy-propyl)valine (diHOPrVal) detached from adducted N-terminal valines in Hb using gas chromatography–tandem mass spectrometry using the N-alkyl Edman method (1996). They initially used this method to address occupational exposure to epichlorohydrin and found significant background levels of the diHOPrVal adduct in control individuals (Landin et al., 1996, 1997). Since G also has an ability to introduce dihydroxypropyl groups onto nitrogens in DNA and protein, and G formation in tobacco smoke and heated food components has been indicated, they hypothesized that G was responsible for the background diHOPrVal adduct. Their further studies demonstrated elevated levels of diHOPrVal adducts in tobacco smokers (vs. non-smokers), and in rats fed a fried diet (vs. a standard diet) substantiating their hypothesis (Landin et al., 2000).

Even after the company halted marketing of DAG oil, many consumers including company employees voluntarily continued to use the remaining DAG oil for their cooking at home. Furthermore, food cooked using the DAG oil was served in the company cafeteria for a few months before running out of stock. Through a survey on diet practice among the company employees we could identify personnel who had ingested relatively high or low levels of DAG oil over a period of 4 months (120 days).

In the present study, we measured diHOPrVal adduct levels in human Hb as a biomarker for G exposure, and compared adduct levels between DAG oil users and non-users. Our data address whether there is any evidence of increased G exposure owing to the ingestion of DAG oil containing small amount of GEs.

## 2. Materials and methods

### 2.1. Chemicals

All chemicals and solvents used were of analytical grade. All glassware used for derivatization of globin samples was silanized with 2% dichlorodimethyl silane in 1,1,1-trichloroethane (Wako Pure Chemicals, Osaka, Japan).

### 2.2. Instrumentation and conditions

A 1200 tandem quadrupole mass spectrometer coupled to a CP-3800 gas chromatograph (Bruker Daltonics, California, USA) was used for GC–MS/MS analysis. The gas chromatographic analysis was performed with a DB-5MS fused silica capillary column (Agilent Technologies, California, USA) (l. = 30 m, i.d. = 0.25 mm, f.t. = 0.25 μm). Ultrapure helium with a constant flow of 1.5 mL/min was used as carrier gas. The injection volume was 2 μL (splitless), the injector temperature was 280 °C, and the mass interface temperature was set at 230 °C. The oven program was 1 min at 100 °C, 20 °C/min to 240 °C, and 10 °C/min to 300 °C. Ions were formed for mass spectrometric detection using negative ion chemical ionization (NCI). For NCI, methane was used as the reagent gas at a filament emission current of 150 μA, electron energy of 70 eV and ion source temperature of 150 °C. Multiple reaction monitoring (MRM) was used to monitor precursor to product ion transition of *m/z* 379 → 277, 295 and 337 for diHOPrVal-PFPTH, and *m/z* 384 → 282, 300 and 342 for d5-diHOPrVal-PFPTH.

### 2.3. Standards

Standards (N-(2,3-dihydroxypropyl)-DL-valine (diHOPrVal), N-(2,3-dihydroxy ($^2H_5$)propyl)-DL-valine (d5-diHOPrVal), dihydroxypropyl-globin (diHOPrGlobin) and dihydroxy($^2H_5$)propyl-globin (d5-diHOPrGlobin)) used for the measurement of diHOPrVal adduct were all synthesized or prepared internally (Kao Corporation).

Alkylated globin (diHOPrGlobin and d5-diHOPrGlobin) was used as standard and internal standard respectively for determination of adduct levels in samples, prepared by adding G or d5-G to hemolysate (Landin et al., 1996, 2000). The levels of diHOPrVal adducts in the standard globin were determined through comparison with standard diHOPrVal using d5-diHOPrVal as internal standard after derivatization and GC–MS/MS analysis according to Landin et al. (1996). However, we did not employ total hydrolysis treatment before N-alkyl Edman reaction because this treatment is insensitive, time-consuming, and susceptible to artifact formation. Although efficiency of derivatization between free valine (amino acid) and globin (protein) is different, possibly resulting in errors in absolute levels of adduct (Törnqvist et al., 1992), we considered the potential impact on our objective, exposure comparison, as limited. The level of d5-diHOPrVal adducts in the internal standard globin was also determined by the same method (comparison with standard d5-diHOPrVal using diHOPrGlobin, of which adduct level was determined in advance, as internal standard).

### 2.4. Information regarding donors

As described in Section 1, even after the company halted marketing of DAG oil, many consumers including company employees voluntarily continued to use the remaining DAG oil for their cooking at home. Furthermore, food cooked using DAG oil was served in the company cafeteria on working days for a few months before running out of stock. Through a survey on diet practice among the company employees, we identified 7 DAG oil users and 6 non-users. DAG oil users were defined as individuals who had daily used the cafeteria for lunch (over 20 times/month) and also used DAG oil at home for cooking. Non-users were defined as individuals who had seldom used the cafeteria (less than 5 times/month) and who did not use DAG oil for cooking at home. Following procedures regulated by the company ethical committee, blood samples were obtained with written informed consent explaining the objective of the study in advance.

The shelf life of DAG oil had been set at one year. Based on the record of batch analysis, average GEs level of DAG oils shipped during the period of one year plus 120 days (life span of human Hb) before the blood sampling was estimated as 200 ppm (back calculated from a value of 65.7 ppm as 3-monochoropropane-1,2-diol equivalent). All donors were asked to fill a brief-type self-administered diet history questionnaire (BDHQ) (Sasaki, 2004) for understanding their diet habits. In addition, since it has been reported that smoking might affect G-Hb adduct levels (Landin et al., 1997), information regarding smoking habits was obtained.

### 2.5. Blood sample preparation and analysis of hemoglobin adducts

Human blood samples were collected in heparinized vacutainer tubes. Erythrocytes, separated from plasma and washed twice with 1–3 volumes of saline, were then stored at −80 °C. Frozen blood samples were melted by rapid thawing at 37 °C, then were hemolysed by the addition of 1.5 volumes of MilliQ water. Human globin was precipitated with ethyl acetate from an isopropanol/HCl solution of the hemolysate according to Mowrer et al. (1986).

Adducts to N-terminal valine in globin were detached as pentafluorophenylthiohydantoin (PFPTH)-valines through derivatization with pentafluorophenyl isothiocyanate (PFPITC) according to the N-alkyl Edman method (Landin et al., 1996).

Calibration samples were prepared by addition of different amounts of the characterized standard globin (diHOPrGlobin), corresponding to 0–100 pmol/g globin of diHOPrVal.

### 2.6. Statistics

Comparison of diHOPrVal adduct level between DAG oil users and non-users were made using a two-tailed Welch's t-test in Microsoft Office Excel 2003 (Microsoft Corporation), and $p < 0.05$ was considered significant.

## 3. Results and discussion

Data for Hb adducts in the blood samples are shown in Table 1. G-Hb adducts were detected in all samples. The average level of diHOPrVal was 3.5 ± 1.9 pmol/g globin in the DAG oil users and 7.1 ± 3.1 pmol/g globin in the non-users. No increased trend of G-Hb adducts in DAG oil users compared to non-DAG oil users was observed. Based on the survey using BDHQ, assumed ingestion of edible oil of the donors ranged from 1.4 to 9.2 g/day among the non-users (average value is 6.4 ± 2.9 g/day), and from 0.8 to 18.3 g/day among the DAG oil users (average value is 9.4 ± 6.0 g/day).

The outcome of the present study suggests that there was no increased exposure to G in persons who daily ingested DAG oil containing small amounts of GEs. Even if GEs were converted to G in the body, extra exposure to G had a minimal impact relative to the background level. It should be noted that further study to characterize and quantify G-Hb adduct formation is ongoing. G-Hb adduct levels demonstrated in the present study might be further verified. Nevertheless, as all samples were quantified under the same conditions, we concluded that G-Hb adduct levels of the DAG oil users were not higher than those of the non-users.

Landin et al. (1997) also reported similar levels of diHOPrVal in German and Swedish subjects without known chemical exposure.

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