Studies of hemoglobin adducts as biomarkers of human internal exposure to glycidol-related compounds

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Abstract

Glycidol (G) is an alcohol with a highly reactive functional epoxide group. Because animal studies have indicated that G has carcinogenic and genotoxic effects, it is considered likely to have similar effects in humans, although no epidemiological analysis of this issue has been conducted. Recently, concerns about daily exposure to G have grown because of the discovery of glycidol fatty acid esters (GEs) as contaminants of edible oils, and detection of G-hemoglobin (Hb) adducts in individuals without known chemical exposure. In general, risk is defined as the probability that exposure to a hazard will lead to a negative consequence (risk = hazard × exposure). Thus, evaluation of "exposure" to the "hazard" (G) is crucial for assessment of its "risk" to human health. However, direct biomonitoring of extremely low exposure in daily life is difficult to perform, since highly reactive electrophilic compounds react with nucleophilic biomacromolecules (such as proteins and DNA) and therefore have a short half-life in the body.

This study focused on the usefulness of Hb adducts for evaluation of chemical exposure. Levels of Hb adducts correlated with the area under the concentration-time curve (AUC) in a toxicokinetics study, indicating that this may provide a biomarker of *in vivo* dose. In addition, Hb adducts are considered stable over the erythrocyte lifetime, indicating the potential for sensitive detection of accumulated daily chemical exposure. G has been reported to form *N*-(2,3-dihydroxypropyl)valine (diHOPrVal) Hb adducts. However, diHOPrVal formation has only been demonstrated under *in vitro* conditions and it has not been investigated as an *in vivo* exposure marker. We investigated the utility of the published analytical method for Hb diHOPrVal adduct detection as an exposure marker, and then applied this method to evaluate exposure to G-related compounds.

Chapter 1 describes the establishment of a selective and sensitive method for Hb adduct analysis by using a modified Edman method with gas chromatography-negative chemical ionization-tandem mass spectrometry (GC-NCI-MS/MS). Globin samples from rats administered G were analyzed repeatedly to evaluate between-run and within-run reproducibility. Between-run and within-run coefficients of variation (CV) were 8.8% and 12%, respectively, and no significant variance was observed. The lower limit of quantification (LOQ) was 0.6 pmol/g globin, indicating higher sensitivity than that previously reported (Landin *et al.*, 1996), owing to a lower signal-to-noise (S/N) ratio. These data indicated the successful establishment of a sensitive method for quantification of Hb diHOPrVal adducts.

Chapter 2 describes an investigation of the kinetics of diHOPrVal formation and elimination, *in vitro* and *in vivo,* to provide the information necessary to support the use of these adducts as markers of G exposure. Five groups of rats were orally administered single doses of G ranging from 0 to 75 mg/kg body weight, and a dose-dependent increase in diHOPrVal levels was observed 24 h after administration. Blood sampling at different time points (1-40 days) from four groups administered 12 mg/kg body weight G showed that diHOPrVal levels decreased linearly, consistent with normal erythrocyte turnover, indicating diHOPrVal adduct stability. *In vitro* experiments with whole blood were conducted to determine the second-order rate constant (k_{val}) for the reaction of G with the *N*-terminal valine of rat and human Hb. No species differences were identified, because k_{val} was 6.7 ± 1.1 and 5.6 ± 1.3 (pmol/g globin per μMh) for rat and human Hb, respectively. *In vivo* doses estimated from k_{val} and diHOPrVal levels were in agreement with the AUC values measured in a rat toxicokinetics study. The above results indicated that diHOPrVal was a useful biomarker for quantification of exposure to G.

Chapter 3 applied the G-Hb adduct to the evaluation of human exposure to G-related compounds. Diacylglycerol (DAG) oil is unique edible oil that consists primarily of DAG and also contains some glycidol fatty acid esters (GEs). It has previously been marketed

as a Food for Specified Health Uses in Japan. We therefore conducted a DAG oil exposure evaluation by using the diHOPrVal adduct in exposed human subjects, to assess the cancer risk from *in vivo* metabolism of GEs to produce G. This matched case-control study found that the average diHOPrVal levels were 6.9 pmol/g globin (95% CI: 4.9–9.0) for 14 DAG oil-exposed subjects and 7.3 pmol/g globin (95% CI: 6.1–8.5) for 42 non-exposed volunteers, with no significant difference between the two groups. A second study compared the diHOPrVal levels in 12 DAG oil-exposed subjects before and after discontinuing the use of DAG oil, and detected no significant change in the levels of this adduct (from 7.1 ± 1.1 to 7.5 ± 1.4 pmol/g globin). These results suggested that humans who ingested DAG oil daily did not show increased exposure to G. On the other hand, the daily internal G exposure in non-DAG oil-exposed volunteers was estimated to be 22 nMh/day. This suggested that some level of cancer risk was associated with G-related compounds.

This study identified diHOPrVal adduct formation *in vivo* in rats administered G for the first time. It revealed that diHOPrVal was a powerful biomarker for *in vivo* G exposure, showing dose-dependent formation, chemical stability, and sensitivity. In addition, determination of k_{val} facilitated the use of diHOPrVal levels to estimate the *in vivo* dose. Human exposure evaluations indicated that humans without known chemical exposure had detectable levels of diHOPrVal adducts. On the other hand, the diHOPrVal adduct levels in humans who had ingested the DAG oil containing GEs were equivalent to those found in non-exposed subjects, indicating that cancer risk did not increase. These findings indicated that it would be possible to develop a new realistic approach to cancer risk assessment using the daily internal exposure level, derived from diHOPrVal Hb adduct levels. This method has the potential to improve conventional risk assessment, based on the dose ingested, by providing a foundation for the development of novel risk assessments based on actual internal exposure.

Table of contents

Figures

Tables

Abbreviations

ALARA	as low as reasonably achievable
ANOVA	analysis of variance
AUC	area under the concentration-time curve
BDHQ	brief-type self-administered diet history questionnaire
BfR	German Federal Institute for Risk Assessment
BMDL	benchmark dose lower confidence limit
$BMDL_{10}$	benchmark dose lower confidence limit 10%
CEV	*N*-(2-carbamoylethyl)valine
CI	confidence interval
CV	coefficient of variation
DAG	diacylglycerol
diHOPrVal	*N*-(2,3-dihydroxy-propyl)valine
EFSA	European Food Safety Authority
ENU	ethylnitrosourea
ESI	electrospray ionization
FAO	Food and Agriculture Organization
FFQ	food frequency questionnaire
FOSHU	food for specified health uses
G	glycidol
GC	gas chromatography
GEs	glycidol fatty acid esters

GL	glycidol linoleate
Hb	hemoglobin
IARC	International Agency for Research on Cancer
$iBMDL_{10}$	internal benchmark dose lower confidence limit 10%
iDEL	internal daily exposure level
ILSI	International Life Sciences Institute
iMOE	internal margin of exposure
HOEtVal	*N*-2-hydroxyethylvaline
HPLC	high-performance liquid chromatography
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC	liquid chromatography
LOQ	low limit of quantification
MOCA	4,4′-methylenebis(2-chloroaniline)
MOE	margin of exposure
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NCI	negative ion chemical ionization
NMR	nuclear magnetic resonance
NOEL	no-observed-effect-level
NOAEL	no-observed-adverse-effect-level
NTP	National Toxicology Program
PFPITC	pentafluorophenyl isothiocyanate
PFPTH	pentafluorophenylthiohydantoin
POD	point of departure

QC	quality control
SD	standard deviation
S/N	signal-to-noise
WHO	World Health Organization

List of Papers

The thesis is based on the following papers, which are referred to in the text by Roman numerals, I-III. Some unpublished results have also been included in the thesis.

I) **Honda, H.**, Onishi, M., Fujii, K., Ikeda, N., Yamaguchi, T., Fujimori, T., Nishiyama, N., Kasamatsu, T., 2011. Measurement of glycidol hemoglobin adducts in humans who ingest edible oil containing small amounts of glycidol fatty acid esters.
Food Chem. Toxicol., 49, 2536–2540.

II) **Honda, H.**, Fujii, K., Yamaguchi, T., Ikeda, N., Nishiyama, N., Kasamatsu, T., 2012. Glycidol exposure evaluation of humans who have ingested diacylglycerol oil containing glycidol fatty acid esters using hemoglobin adducts.
Food Chem. Toxicol., 50, 4163–4168.

III) **Honda, H.**, Törnqvist, M., Nishiyama, N., Kasamatsu, T., 2014. Glycidol–hemoglobin adducts as biomarkers of exposure and in vivo dose.
Toxicol. Appl. Pharmacol., 15, 213–220.

Other reference works:

Honda, H., Ichiyanagi, K., Suzuki, J., Ono, T., Koyama, H., Kajikawa, M., Okada, N., 2007. A new system for analyzing LINE retrotransposition in the chicken DT40 cell line widely used for reverse genetics.
Gene, 15, 116–124.

Ikeda, N., Fujii, K., Sarada, M., Saito, H., Kawabata, M., Naruse, K., Yuki, K., Nakagiri, H., **Honda, H.**, Tamaki, Y., Nishiyama, N., Kasamatsu, T., 2012. Genotoxicity studies of glycidol fatty acid ester (glycidol linoleate) and glycidol.
Food Chem. Toxicol., 50, 3927–3933.

Takahashi, Y., Koike, M., **Honda, H.**, Ito, Y., Sakaguchi, H., Suzuki, H., Nishiyama, N., 2008. Development of the short time exposure (STE) test: an *in vitro* eye irritation test using SIRC cells.
Toxicol. In Vitro., 22, 760–770.

Watanabe, T., Suzuki, T., Natsume, M., Nakajima, M., Narumi, K., Hamada, S., Sakuma, T., Koeda, A., Oshida, K., Miyamoto, Y., Maeda, A., Hirayama, M., Sanada, H., **Honda, H.**, Ohyama, W., Okada, E., Fujiishi, Y., Sutou, S., Tadakuma, A., Ishikawa, Y., Kido, M., Minamiguchi, R., Hanahara, I., Furihata, C., 2012. Discrimination of genotoxic and non-genotoxic hepatocarcinogens by statistical analysis based on gene expression profiling in the mouse liver as determined by quantitative real-time PCR.
Mutat. Res., 18, 164–175

Introduction

Cancer risk associated with glycidol and glycidol-related compounds

Glycidol (2,3-epoxy-1-propanol, G; Fig. 1) is an important intermediate for the production of functional epoxides and pharmaceuticals. G is a reactive electrophilic molecule that is capable of reacting with larger biomolecules such as DNA and proteins. The toxicological and carcinogenic potential of G has been investigated extensively and has been reviewed under the National Toxicology Program (NTP) and by the International Agency for Research on Cancer (IARC) (NTP, 1990; IARC, 2000). The NTP study showed evidence of carcinogenicity in B6C3F1 mice and F344/N rats. On the basis of this evidence, IARC evaluated G as "probably carcinogenic to humans" (Group 2A), although no relevant human epidemiological data were available.

Recently, G-related-compounds (glycidol fatty acid esters [GEs], Fig. 1), were found in some refined edible oils (such as palm oil), fats, and infant formula preparations. In addition, various food components (anhydro sugars, allyl alcohol, glycerol, glycerol halohydrins, etc.) have been shown to form G, either chemically or by metabolism (Hauschild and Petit, 1956; Hamlet, 1998; Piasecki *et al.*, 1990; Jones, 1975; Patel *et al.*, 1980; Ishidao *et al.*, 2002). This has led to growing concerns about the potential release of G from these potential precursors in foods during digestion. The cancer risk posed by GEs in foods is considered to be the most important issue, since release of G from GEs has been demonstrated in animal studies (Wakabayashi *et al.*, 2011; Appel *et al.*, 2013).

Nevertheless, limited information relating to the carcinogenicity of GEs has been published. One of the GEs, glycidyl (glycidol) oleate, has been tested in a limited study

in mice, where subcutaneous injection produced a low incidence of local sarcomas. Glycidol stearate was also tested in mice, indicating that subcutaneous injection produced no significant increase in the incidence of local tumors (Walpole, 1958). IARC previously examined these results and judged that they did not provide sufficient evidence to reach firm conclusions about the carcinogenicity of this compound. Accordingly, glycidyl oleate and glycidyl stearate were classified as Group 3, indicating that they were not classifiable with regard to human carcinogenicity (IARC, 1976a,b, 1987).

Ikeda *et al.* (2012) evaluated the genotoxic potential of glycidol linoleate (GL) and G, using three established genotoxicity tests (a bacterial reverse mutation test, an *in vitro* chromosomal aberration test, and an *in vivo* bone marrow micronucleus test). In the bacterial reverse mutation test, GL and G showed positive responses. The positive responses of GL were fewer than those of G, and were only observed in strains showing point mutations, whereas G showed remarkably positive responses. G was involved in the positive response of GL. GL did not induce chromosomal aberrations, whereas G induced them in the presence and absence of metabolic activation. In the bone marrow micronucleus test, neither GL nor G induced significant increases in micronucleated immature (polychromatic) erythrocytes. Based on the above results, as well as pertinent toxicokinetic information, GL did not have significant genotoxic activity.

In addition, our follow-up study using GL to investigate GE toxicokinetics revealed that G, but not GL, was quantifiable in the plasma of animals (rats and monkeys) that were orally administered a high dose of GL (341 mg/kg). This result suggested that GEs were not bioavailable, and characterizing G exposure may be necessary for the assessment of the health risk presented by GEs in foods. Recently, Appel *et al.* (2013)

published a kinetic study of [^{14}C]glycidyl [^{3}H]palmitate ester. This study indicated that G-derived radioactivity was excreted primarily in the urine, while palmitate-derived radioactivity was primarily excreted in the feces. This result supported our hypothesis that GEs were hydrolyzed and circulated in the blood, whilst other parts of fatty acid esters are systemically unavailable. Thus, evaluation of G exposure is essential for understanding the cancer risk in individuals exposed to GEs.

Diacylglycerol oil and glycidol fatty acid esters

Diacylglycerol (DAG) oil is a unique edible oil that is defined as containing at least 80% (w/w) DAG. DAG oil has been shown to reduce body fat accumulation, and has previously been marketed in Japan by the Kao Corporation, as a Food for Specified Health Uses (FOSHU) (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 GEs in refined edible oils, because of the potential release of G from GEs during digestion in humans (BfR, 2009a,b; Bakhiya *et al.*, 2011). GEs are produced 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, but nonetheless at levels considerably higher than those detected in other commercial edible oils. According to a report by Masukawa *et al.* (2010), DAG oil contained 269 μg/g GEs, whereas two commercial edible oils that mainly consisted of triacylglycerol contained significantly lower GE concentrations (22.8 μg/g and 6.7 μg/g). These concentrations represented the summed value of each GE present, i.e., C16:0-GE, C18:0-GE, C18:1-GE, C18:2-GE, and C18:3-GE. The most

common GE present in DAG oil was GL (132 μg/g), followed by glycidol oleate (96 μg/g), with minor amounts (6.0 μg/g) of glycidol palmitate. GL (9.0 μg/g) and glycidol oleate (10.2 μg/g) were identified as the major GEs in one commercial edible oil that primarily consisted of triacylglycerol. As a result of these findings, 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 the BfR stated, there was no suitable analytical method to analyze the actual exposure levels for GE and G, and little information was available to address the issue of the potential conversion of GEs into G in the human body.

Exposure evaluation using hemoglobin adducts

In general, risk is assessed by the multiplication of hazard by exposure. Therefore, evaluation of exposure to the relevant chemical is required in order to draw conclusions about the human health risk. Traditional exposure evaluations employ food frequency questionnaires (FFQs) to estimate the dose ingested. However, in general, toxicity is related to internal exposure (corresponding to the area under the concentration-time curve [AUC] in a toxicokinetic study) rather than the ingestion dose. In addition, FFQ-based estimation of ingestion dose is not entirely precise. Therefore, traditional exposure evaluation can be improved by the analysis of exposure biomarkers that reflect internal exposure.

We therefore focused on the use of hemoglobin (Hb) adducts as an internal exposure marker of G, in order to assess and/or monitor internal exposure to various reactive chemicals (Törnqvist and Landin, 1995; Törnqvist *et al.*, 2002; Boogaard, 2002; Ogawa

et al., 2006). The application of Hb adduct measurement for evaluation of exposure is shown in Fig. 2. Compared with other classical biomarkers, such as the target chemicals and their metabolites in blood and urine, Hb adducts have some advantages.

- Since Hb adducts are stable for 123 days in humans, *in vivo* doses are integrated over a long and well-defined time period, allowing for sensitive detection (Osterman-Golkar *et al.*, 1976).
- Large amounts of Hb are easily available in exposed animals and humans.
- Differences between species and between individuals with regard to metabolism, absorption, and uptake rate are reflected in the levels of Hb adducts formed *in vivo*, thus providing a basis for extrapolation between species and dosimetry-based risk assessment.

For these reasons, Hb adduct evaluation provides a valid method to estimate the very low exposure to G related to the ingestion of GEs.

The G-Hb adduct - *N*-(2,3-dihydroxypropyl)valine

G has been reported to form Hb adducts. Landin *et al.* demonstrated sensitive detection of *N*-(2,3-dihydroxypropyl)valine (diHOPrVal), detached from adducted Hb *N*-terminal valines by using the *N*-alkyl Edman method (1996) and gas chromatography-tandem mass spectrometry (GC-MS/MS). They initially used this method to investigate 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 has been indicated in tobacco smoke and heated food components, they hypothesized that G was responsible for these background diHOPrVal adducts. Their subsequent studies demonstrated elevated levels of diHOPrVal adducts in tobacco smokers (versus non-smokers), and in rats fed a fried diet (versus a standard diet), substantiating their hypothesis (Landin *et al.*, 2000). However, diHOPrVal formation has been only proved under *in vitro* conditions and it has not been characterized as an *in vivo* exposure marker. Therefore, before the diHOPrVal adduct could be used to evaluate human exposure, further characterization of its relationship with G exposure is required.

Aims of the thesis

The central aim of this thesis was to evaluate the characteristics of the G-Hb adduct as a potential *in vivo* marker of G exposure, and to apply this marker to the evaluation of human G exposure following ingestion of DAG oil containing GEs.

The specific G-Hb adduct characterization aims were to:

- Establish the modified Edman method for the measurement of G-Hb adducts using gas chromatography-negative chemical ionization-tandem mass spectrometry (GC-NCI-MS/MS).
- Characterize the relationship between G dose and Hb adduct formation, and adduct chemical stability *in vivo*.
- Evaluate the *in vivo* dose predictivity of G-Hb adducts via determination of the second-order reaction rate constant.

The specific human exposure evaluation and risk assessment aims were to:

- Statistically evaluate the effects of DAG oil ingestion and subject characteristics (sex and age) on G-Hb adduct levels in a matched case-control study.
- Evaluate the effect of discontinuing DAG oil on the G-Hb adduct levels.
- Assess the relative risks of DAG oil ingestion and daily environmental exposure.

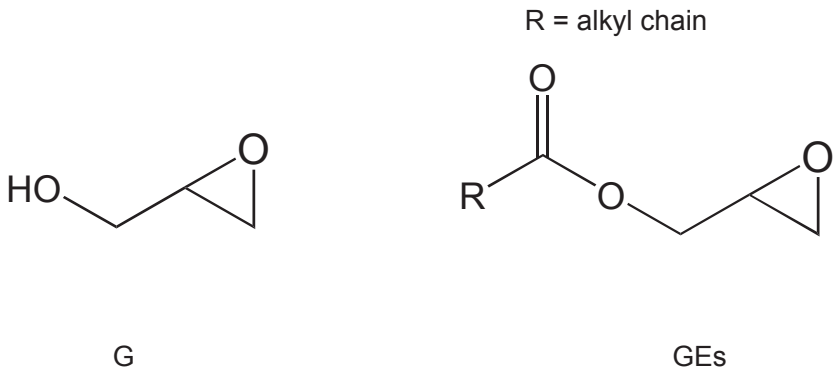

Fig. 1 Structures of glycidol (G) and glycidol fatty acid esters (GEs)

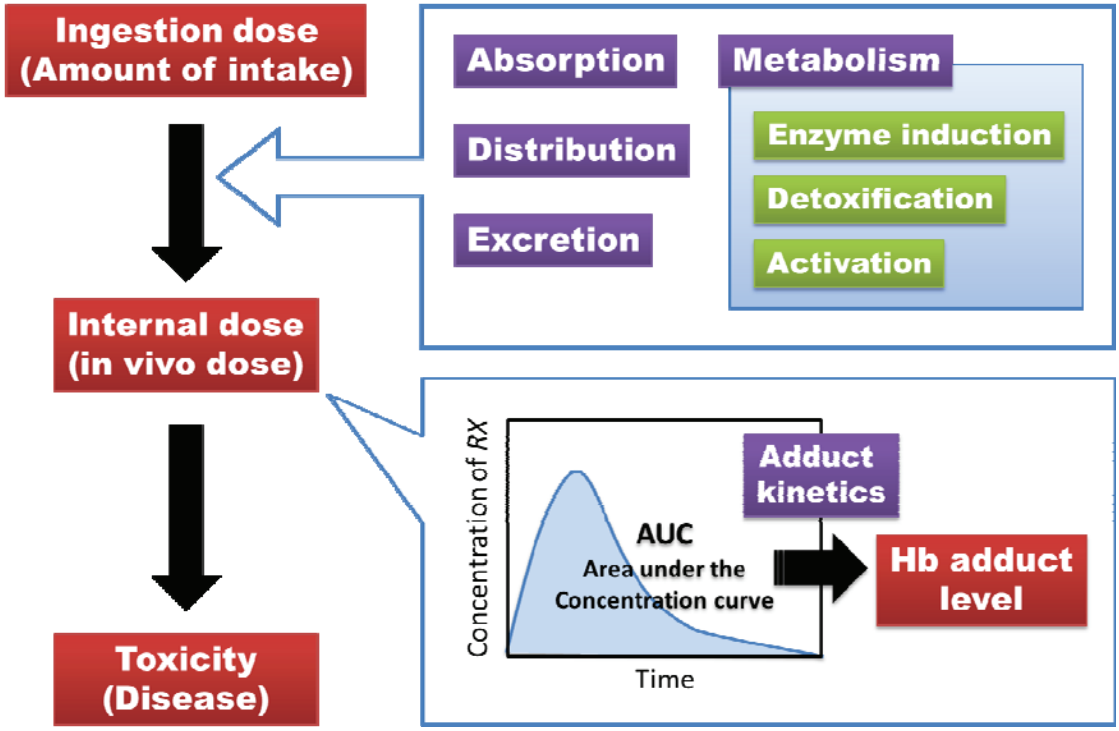

Fig. 2 Application of Hb adducts that reflect internal dose for exposure evaluation

RX = electrophilic agent.

Hb adduct background

Measurement of reactive compounds

Electrophilic compounds react with nucleophilic biomacromolecules such as proteins and DNA. This inherent reactivity can complicate their measurement, because they have short half-lives in the body. Therefore, exposure to such compounds is often evaluated by measuring their stable reaction products with biomacromolecules *in vivo*. Fig. 3 shows the lifespans of urinary metabolites, DNA adducts, and Hb adducts. DNA adducts can be measured in various tissues, and this can provide valuable information regarding the biologically effective dose for a specific tissue and genotoxicity mechanism. However, these adducts generally have short half-lives and complex elimination kinetics because of the range of DNA repair systems involved in their removal. For these reasons, their suitability is limited to the assessment of exposures occurring over the previous few days, up to a maximum of 1 week. Urinary metabolites have an even shorter turnover, which limits their suitability to studies of exposures occurring within the previous 1-2 days.

In contrast, protein adducts (including Hb adducts) reflect exposure over a long time period, because these usually remain stable for the lifetime of the protein. In humans, the half-life of serum albumin is ~19 days and the lifespan of Hb is ~123 days (Furne *et al.*, 2003; Iwao *et al.*, 2009). Mass spectrometric (MS) analyses have been used for the measurement of protein adducts since Ehrenberg *et al.* (1974) suggested the use of blood protein adducts to measure the *in vivo* dose of electrophiles. These studies have primarily focused on exposure to low molecular-weight compounds, either in

experimental animals or in exposed humans. Protein adduct measurements have also been applied to identify background exposure levels in the general population. Several reviews regarding protein adducts have been published (Törnqvist and Landin, 1995; Törnqvist *et al.*, 2002; Boogaard, 2002; Ogawa *et al.*, 2006).

An example of Hb adduct usage

The first methodologies for Hb adduct measurement were developed for biomonitoring of ethylene oxide exposure in humans (Calleman *et al.*, 1978). Since then, Hb adducts have been used to evaluate exposure to reactive electrophilic chemicals (e.g., alpha-beta-unsaturated compounds, aromatic amines, epoxides, isocyanates, nitro arenes, and organic acid anhydrides) (Calleman *et al.*, 1994; Farmer *et al.*, 1996; Lindh and Jönsson, 1998; Ogawa *et al.*, 2006; Richter and Branner, 2002; Sabbioni and Jones, 2002).

Hb adducts have also been useful for the evaluation of chemical exposure after industrial accidents. One of best-known examples occurred in 1997 in Sweden, when an acrylamide-containing grouting agent used in the construction of a railway tunnel was found to be responsible for the intoxication of workers and residents in connection with the construction of a railway tunnel. The exposure levels were determined by measurement of an acrylamide Hb adduct by using an *N*-alkyl Edman method (Hagmar *et al.*, 2001). In this context, the ability to measure Hb adduct levels weeks or months after the accident represented a considerable advantage.

Hb adducts as an *in vivo* dose marker

The concept of *in vivo* dose (*D*) is a very useful one when considering precise risk assessment or molecular epidemiological studies (see Fig. 2). *D* corresponds to the internal dose, or the AUC in toxicokinetic studies, and is expressed in Mh. In other words, *D* is the product of the concentration of the alkylating agent (*C*) and the exposure time (*t*) (equation 1).

$$D = C \times t \qquad (1)$$

Furthermore, the blood *in vivo* dose of an electrophile could be calculated from Hb adduct levels (*A*) measured a short time after acute exposure, by using the second-order reaction rate constant for the formation of the specific adduct (Ehrenberg *et al.*, 1983) at the Hb *N*-terminal valine (k_{val}) (equation 2a).

$$D = A / k_{val} \qquad (2a)$$

Kinetics of Hb adduct formation and elimination

Hb adducts generated by electrophilic compounds able to form stable (covalent bond) reaction products with Hb are considered to persist for the erythrocyte lifetime. Because this is ~4 months (Furne *et al.*, 2003), Hb adduct levels reflect the net result of adduct formation and removal over the 4 months prior to sampling.

As indicated by equation 2a, the levels of Hb *N*-terminal valine adducts (*A*) (in mol/g

globin) could be determined by multiplying the blood *in vivo* electrophile dose (D) by the second-order reaction rate constant (k_{val}) (in mol/g globin per Mh) calculated a short time after acute exposure (equation 2b).

$$A = D \times k_{val} \qquad (2b)$$

In the same way, the accumulated adduct level (A_{acc}) during long-term exposure is calculated by equation 3 (Fennell *et al.*, 1992; Granath *et al.*, 1992),

$$A_{acc} = A_d \times t\left(1 - \frac{t}{2t_{er}}\right), \quad t < t_{er} \qquad (3)$$

where A_d is the daily adduct level increment and t_{er} is the erythrocyte lifetime.

During chronic exposure to an electrophile that forms stable Hb adducts, the daily adduct level increment (A_d) results in an accumulated adduct level that depends on the erythrocyte lifetime (t_{er}). When the time of continuous exposure (t) is longer than t_{er} ($t \geq t_{er}$), a steady-state level (A_{ss}) is reached, according to equation 4 (Fennell *et al.*, 1992; Granath *et al.*, 1992).

$$A_{ss} = A_d \times \frac{t_{er}}{2}, \quad t \geq t_{er} \qquad (4)$$

The daily *in vivo* dose (corresponding to the internal daily exposure level: *iDEL*) can be calculated from the daily adduct level increment (A_d) when the second-order reaction rate constant for adduct formation is known according to equation 5 (Ehrenberg *et al.*,

1983).

$$iDEL = \frac{A_d}{k_{val}} \qquad (5)$$

The elimination of Hb adducts from the circulation is primarily determined by normal erythrocyte turnover (t_{er}), with a loss of A_0/t_{er} per day. The elimination of Hb adducts can be calculated from the first-order elimination rate constant of the Hb adduct (k_{el}), and the erythrocyte lifetime (t_{er}), using equation 6 (Fennell *et al.*, 1992; Granath *et al.*, 1992),

$$A_t = A_0(1-\frac{t}{t_{er}})e^{-k_{el}t} \qquad (6)$$

where A_o is the starting concentration of the Hb adduct and A_t is the adduct concentration at time t. In a situation where there is considerable animal growth between the time of exposure and blood sampling, there will be an apparent dilution of adduct levels because of the increased blood volume. This "elimination" could be included in k_{el} in equation 6. In this study, we measured the increase in rat body weight (bw) at the sampling points and included this as a separate term in equation 7 (Osterman-Golkar *et al.*, 1998).

$$A_t = A_0(1-\frac{t}{t_{er}})e^{-k_{el}t}\frac{bw_0}{bw_t} \qquad (7)$$

Methodology for measurement of Hb adducts and the *N*-alkyl Edman method

There are two main approaches to the measurement of Hb adducts. Analysis of the adduct can be conducted after its detachment from the amino acid residue, or the modified amino acid can be analyzed after cleaving the peptide bond. Methods involving adduct detachment from the amino acid residues will generally give a result that reflects adducts formed at all sites in the Hb molecule, while the methods involving specific cleavage of a modified amino acid or peptide reflect adduct formation at that particular site. The latter approach provides higher specificity.

The *N*-alkyl Edman method for analysis of Hb adducts represented a breakthrough with regard to adduct biomonitoring because it provided a mild, sensitive, and relatively versatile method (Törnqvist *et al.*, 1986). The principle underlying the normal Edman method and its modification are shown in Fig. 4 and Fig. 5, respectively. Classical Edman degradation requires acidic conditions during the derivatization step to detach the unmodified amino acids after reaction with the Edman reagent, phenyl isothiocyanate (PITC) (Fig. 4). However, when this approach was tested for the detachment of *N*-terminal valine Hb adducts, they were found to detach under neutral conditions (Jensen *et al.*, 1984) (Fig. 5). This observation provided the basis for the development of the modified *N*-alkyl Edman method using perfluorinated Edman reagent (PFPITC) (Törnqvist *et al.*, 1986). The use of this fluorinated reagent afforded improved sensitivity for analysis using GC-MS in the negative ion chemical ionization (NCI) mode. This method has been used for the measurement of several alkylating agents such as ethylene oxide, acrylamide, G and other low molecular-weight electrophiles. Although the reason for the selective detachment of adducted *N*-terminal valines was unclear at the time, it was subsequently discovered that this could be

explained by the "gem-dialkyl effect" (Rydberg *et al.*, 2002), whereby cyclization and detachment of the substituted molecules (those with adducts) are enhanced.

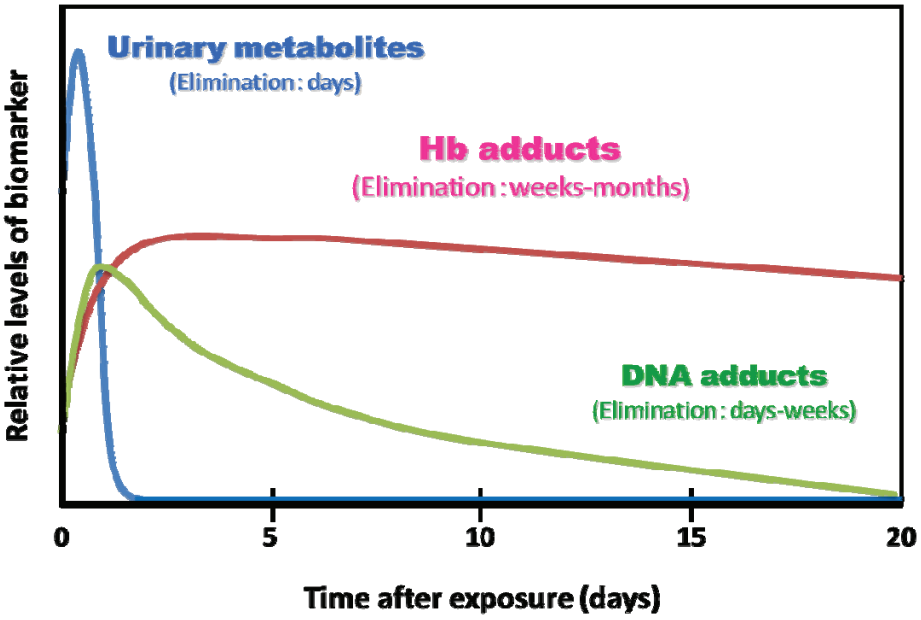

Fig. 3 Biomarker stability following a single administration at time zero

This figure was generated using information published by Stedingk *et al.* (2012).

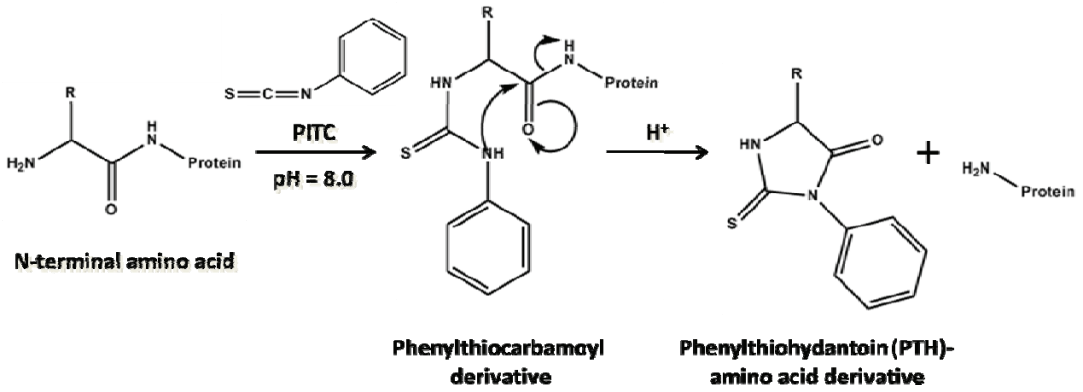

Fig. 4 The classical Edman method for determination of *N*-terminal amino acids

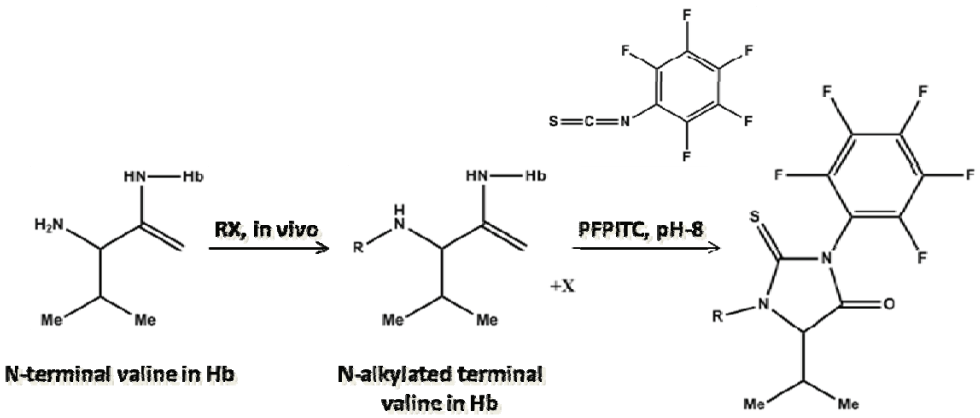

Fig. 5 The modified Edman method for determination of the Hb alkylated *N*-terminal valine

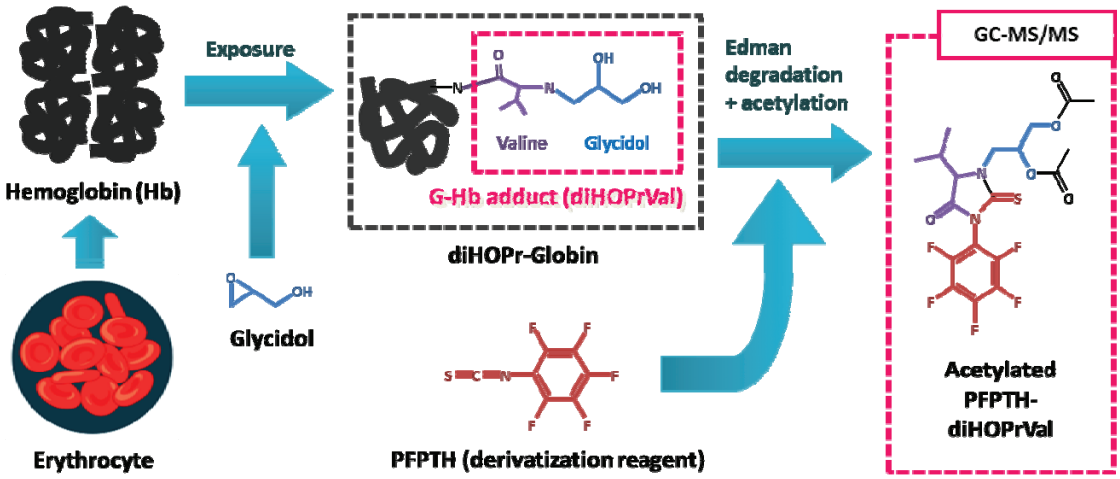

Fig. 6 Determination of G-Hb adducts using the modified Edman method

Carcinogen risk assessment background

Margin of exposure

The margin of exposure (MOE), obtained by dividing the benchmark dose lower confidence limit 10% ($BMDL_{10}$) by the daily exposure levels, is widely known as a tool for human cancer risk evaluation.

$$MOE = \frac{BMDL_{10}}{daily\ exposure\ levels} \qquad (8)$$

In 2005, the Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) and the European Food Safety Authority (EFSA) developed risk assessment guidance for genotoxic carcinogens (FAO/WHO, 2005; EFSA, 2005). Carcinogenic processes mediated via a DNA-reactive mode of action (MOA) are not considered to have thresholds for the dose-response relationship. Therefore, the limit of detection corresponds to the no-observed-effect-level (NOEL) of the carcinogenicity test. Consequently, it has been recommended that exposure to compounds with this MOA should be reduced to as low as reasonably achievable (ALARA). However, such advice was considered to be of limited value, because it did not take human exposure or carcinogenic potency into account. Thus, JECFA concluded that the MOE was the preferred approach for cancer risk assessment of genotoxic carcinogens (FAO/WHO, 2005). The EFSA also concluded that the use of MOE by risk managers for priority setting was more informative than recommending that exposure should be reduced to ALARA (EFSA, 2005). Subsequently,

the MOE was defined as the ratio of the no-observed-adverse-effect level (NOAEL), or BMDL, for the critical effect to the theoretical, predicted, or estimated exposure dose or concentration (equation 8) (WHO, 2009). Because it is not considered scientifically valid to identify an NOAEL for carcinogens, the MOE is calculated from a point of departure (POD), such as BMDL. Finally, the EFSA/WHO/ILSI (International Life Sciences Institute) conference concluded that the MOE approach provided a useful and pragmatic option for risk assessment of genotoxic carcinogens, since it allowed comparisons to be made between compounds, in order to support prioritization in risk management.

Internal MOE

Although the magnitude of the MOE gives an indication of the level of concern, as the larger the MOE, the smaller the potential risk, it does not provide a precise quantification of risk. One reason for this relates to the margin of difference between the effects of a human dose and the equivalent dose administered in animal experiments, due to species differences in disposition (such as absorption and metabolism). If we can calculate the margin between the internal exposure dose in humans and the AUC generated from an animal experiment, it will help provide a more precise estimation of the risk to human health.

The internal MOE (iMOE) provides a novel risk assessment tool for carcinogens, calculated by dividing the internal dose at $BMDL_{10}$ ($iBMDL_{10}$) by *iDEL* (equation 9). Because *iDEL* is usually very low, it is unavailable for classical biomarkers such as target chemicals and their metabolites in blood and urine. Using Hb adduct levels (see

equations 4 and 5), *iDEL* for chronic exposure (t [exposure time] ≥ t_{er} [lifetime of erythrocyte]) can be estimated by dividing the accumulated adduct levels (A_{ss}) by half of t_{er} (equation 10). Although iMOE can only be applied to a limited number of compounds at the moment, it can enable improved human risk assessment.

$$iMOE = \frac{iBMDL_{10}}{iDEL} \qquad (9)$$

$$iDEL = \frac{2A_{ss}}{t_{er}}, \quad t \geq t_{er} \qquad (10)$$

Chapter 1. Establishment of a method for G-Hb adduct measurement

1.1 Introduction

Quantification of G-Hb adducts using a modified Edman method was reported by Landin *et al.* (1996). At the beginning of this investigation, we established this method in our laboratory (Landin *et al.*, 1996, 1997) and investigated the analytical sensitivity and reproducibility of the results obtained. The procedure used for diHOPrVal measurement is illustrated in Fig. 6, and the materials and methods employed are mainly presented in Papers I and III (Honda *et al.*, 2011; 2014).

1.2 Materials and methods

1.2.1 Chemicals

Pentafluorophenyl isothiocyanate (PFPITC) and G (purity: 96%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). L-Valine methyl ester hydrochloride was obtained from Acros Organics (New Jersey, USA). Tripotassium phosphate was obtained from Wako Pure Chemicals (Osaka, Japan). Sodium hydrogen carbonate and *t*-butyl alcohol were obtained from Kanto Chemicals (Tokyo, Japan). ($^{2}H_5$)Epichlorohydrin was obtained from CDN Isotopes (Quebec, Canada). All other 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).

1.2.2 Instrumentation and conditions

A Varian 1200 tandem quadrupole mass spectrometer (Bruker Daltonics, California, USA) coupled to a CP-3800 gas chromatograph (Bruker Daltonics, California, USA) was used for GC-MS/MS analysis. The GC analysis was performed with a DB-5MS fused silica capillary column (length = 30 m, internal diameter = 0.25 mm, film thickness = 0.25 µm) (Agilent Technologies, California, USA). Ultrapure helium with a constant flow of 1.5 mL/min was used as the 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, increasing by 20°C/min to 240°C, and by 10°C/min to 300°C. Mass spectrometric detection using negative ion chemical ionization (NCI) was applied. 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-pentafluorophenylthiohydantoin (PFPTH), and *m/z* 384 → 282, 300, and 342 for *N*-(2,3-dihydroxy($^2H_5$)propyl)-L-valine (d5-diHOPrVal)-PFPTH. ^{1}H NMR and ^{13}C NMR spectra (600 MHz) were obtained in D_2O-CD_3OD on a Bruker AVANCE III instrument. Liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) analysis was performed on a Bruker Esquire 3000 Plus connected to an Agilent Series 1100 HPLC system.

1.2.3 Standards

1.2.3.1 Synthesis of ($^2H_5$)G

($^2H_5$)Epichlorohydrin (1.05 g, 10.8 mmol) was hydrolyzed to dihydroxy($^2H_5$)-propyl chloride with deionized water (30 mL) at 100°C for 10 h, as described previously (Wang *et al.*, 2008). The reaction mixture was concentrated to obtain the crude product. After evaporation to dryness, the residue was dissolved in chloroform (30 mL). ($^2H_5$)G was then synthesized via addition of tripotassium phosphate (2.44 g) to the dihydroxy($^2H_5$)-propyl chloride solution. The mixture was stirred for 3 h at 55°C (As Tech Corporation, 2008). After filtration, the reaction mixture was evaporated under reduced pressure. Purification by silica gel flash column chromatography gave 0.574 g of ($^2H_5$)G, representing a 67% yield.

1.2.3.2 Synthesis of diHOPrVal

A mixture of L-valine methyl ester hydrochloride (5.00 g, 30 mmol), sodium hydrogen carbonate (2.52 g, 30 mmol), *t*-butyl alcohol (60 mL), and deionized water (15 mL) was heated in an oil bath at 95°C. A solution of G (5.50 g, 74 mmol) in tetrahydrofuran (30 mL) was added dropwise for 1.5 h. After a further 3 h, the reaction mixture was subjected to vacuum concentration to provide the crude product. The residue was dissolved in water (15 mL), and sodium hydroxide (1.43 g) dissolved in water (15 mL) was added. Ninety minutes later, the mixture was acidified to pH 5.0 by addition of 2 N hydrochloric acid solution. This mixture was evaporated under reduced pressure. Purification was carried out by high-performance liquid chromatography (HPLC) using an Inertsil ODS3 column (2.0 × 25 cm) with a mobile phase comprising 0.1% formic acid/2% acetonitrile/water, and UV detection at 220 nm. This resulted in 1.62 g of diHOPrVal, representing a 28% yield.

^{1}H-NMR δ 1.07-1.15 (m, 6H, -C$\underline{H}_3$), 2.31-2.40 (m, 1H, $(CH_3)_2C\underline{H}$-), 3.10-3.18 (m, 1H, N-C$\underline{H}_2$-), 3.28-3.35 (m, 1H, N-C$\underline{H}_2$-), 3.63-3.75 (m, 3H, N-C$\underline{H}$-CO_2H, -C$\underline{H}_2$-OH), 4.08-4.14 (m, 1H, > CH-OH).

^{13}C-NMR δ 16.81 (-$\underline{C}H_3$), 17.08 (-$\underline{C}H_3$), 17.89 (-$\underline{C}H_3$), 18.17 (-$\underline{C}H_3$), 28.91 ($(CH_3)_2\underline{C}$H-), 29.07 ($(CH_3)_2\underline{C}$H-), 49.45 (N-$\underline{C}H_2$-), 49.99 (N-$\underline{C}H_2$-), 63.21 (-$\underline{C}H_2$-OH), 63.33 (-$\underline{C}H_2$-OH), 66.56 (> $\underline{C}$H-CO_2H), 67.28 (> $\underline{C}$H-CO_2H), 67.44 (> $\underline{C}$H-OH), 68.29 (> $\underline{C}$H-OH), 172.08 (-$\underline{C}O_2H$).

LC-MS *m/z* = 192 $[M + H]^+$.

1.2.3.3 Synthesis of d5-diHOPrVal

This compound was prepared as described in section 1.2.3.2, except that ($^2H_5$)G was used, instead of G.

^{1}H-NMR δ 0.99-1.08 (m, 6H, -C$\underline{H}_3$), 2.21-2.32 (m, 1H, $(CH_3)_2C\underline{H}$-), 3.52-3.60 (m, 1H, N-C$\underline{H}$-CO_2H).

^{13}C-NMR δ18.0 (-$\underline{C}H_3$), 18.3 (-$\underline{C}H_3$), 19.0 (-$\underline{C}H_3$), 19.2 (-$\underline{C}H_3$), 30.0 ($(CH_3)_2\underline{C}$H-), 30.2 ($(CH_3)_2\underline{C}$H-), 68.9 (> $\underline{C}$H-CO_2H), 69.8 (> $\underline{C}$H-CO_2H), 173.6 (-$\underline{C}O_2H$).

LC-MS *m/z* = 197 $[M + H]^+$.

1.2.3.4 Standard globin preparation

Dihydroxypropyl-globin (diHOPrGlobin) with a known level of adduct was generated to be used as a calibration standard for the determination of diHOPrVal levels in blood sample Hb. diHOPrGlobin was prepared by adding 27 µL of G to 10 mL of hemolysate (5

mL of red blood cells in 1 mM phosphate buffer, pH 7.4), resulting in a final G concentration of 40 mM. This was incubated overnight with agitation at 37°C as described by Landin *et al.* (1996). After globin isolation, the degree of alkylation was determined using the modified Edman method described in Paper II (Honda *et al.*, 2012). In this analysis, diHOPrVal was used as the standard and d5-diHOPrVal (see below) was used as the internal standard. The degree of alkylation was 8800 nmol diHOPrVal per g globin.

1.2.3.5 d5-Dihydroxypropyl-globin internal standard preparation

The d5-dihydroxypropyl-globin (d5-diHOPrGlobin) internal standard was prepared from d5-G synthesized from d5-epichlorohydrin as described in section 1.2.3.1. d5-G (0.25 mL) was added to hemolysate (5 mL), and Hb was alkylated for 24 h at 37°C with agitation. Globin was precipitated by adding the mixture containing hemolyzed erythrocytes dropwise into cold acetone/HCl (1%) while stirring (Anson and Mirsky, 1930). The precipitate was washed with acetone and with diethyl ether, and finally dried overnight at room temperature. To determine d5-diHOPrVal levels, calibration was performed using the synthesized d5-diHOPrVal (see section 1.2.3.3). Standard globin with known amounts of diHOPrVal (see section 1.2.3.4) was used as an internal standard in this analysis.

1.2.3.6 Production of acetylated PFPTH of diHOPrVal or d5-diHOPrVal

During the analysis of standard globin and internal standard globin, acetylated

PFPTH of diHOPrVal and d5-diHOPrVal were produced by derivatization, as described in section 1.2.5.

1.2.4 In vivo G exposure and globin sample preparation in rats

Six-week-old male Sprague-Dawley rats were obtained from Charles River Laboratories Japan, Inc. (Yokohama, Japan) and kept on a 12-h light/dark cycle with free access to food and water. G was dissolved in distilled water and administered by oral gavage to 7-week-old rats in a volume of 5 mL/kg bw. Control animals were given vehicle (water) only. Three groups of rats (n = 3 per group) were administered 0, 0.0164, or 75 mg/kg bw G, and blood was drawn 24 h after administration. Erythrocytes were separated from plasma, washed twice with 1–3 volumes of saline, and then stored at -80°C. Frozen erythrocytes were thawed rapidly at 37°C, and further hemolyzed by addition of 1.5 volumes of MilliQ water. Rat globin was precipitated from an isopropanol/HCl solution of the hemolysate using ethyl acetate (Mowrer, 1986; Törnqvist, 1994). Finally, all globin samples were mixed homogeneously to provide a large quantity of globin with the same amount of diHOPrVal to establish the analytical method. Similarly, globin samples were obtained from three male Sprague-Dawley rats that had not been exposed to G.

1.2.5 Derivatization of globin samples

Derivatization was performed essentially as described by Törnqvist *et al.* (1986), with modifications described by Landin *et al.*, 1996 and Landin *et al.*, 2000 for the analysis of

Hb diHOPrVal adducts. These included the use of d5-diHOPrGlobin as the internal standard, the addition of water before extraction, and the acetylation of the hydroxyl groups in the adduct (Landin *et al.*, 1996). Globin (50 mg) was dissolved in 1.5 mL of formamide, before adding 50 µL of the internal standard globin solution (0.17 nmol/mL) and 8 µL of PFPITC. The derivatization reaction was carried out at room temperature overnight with agitation. Finally, to increase the yield, the reaction mixture was incubated in a shaking water bath at 45°C for 1.5 h. Water (1.5 mL) was then added and the PFPTHs formed were isolated by extraction with diethyl ether, followed by a washing procedure to eliminate by-products. The combined (3 + 3 + 2 mL) ether extract was evaporated under nitrogen. After dissolution in 1 mL of toluene, the sample was washed twice with water (2 × 2 mL) for 10 min with agitation, and twice with freshly prepared 0.1 M disodium carbonate (2 × 3 mL) for 5 min to hydrolyze by-products, before washing again with water (2 mL). The toluene and volatile hydrolyzed by-products were carefully evaporated under nitrogen at 60°C. The dry PFPTH-diHOPrVal was acetylated with 12.5% (v/v) acetic anhydride in acetonitrile in the presence of 12.5% (v/v) triethylamine for 15 min as described by Landin *et al.* (1996), and evaporated under nitrogen at 30°C. The samples were finally re-dissolved in 50 µL of toluene and analyzed by GC-NCI-MS/MS. The product ions *m/z* 337, 295, and 277, formed from the precursor ion of diHOPrVal-PFPTH (*m/z* 379), were measured. The details of the analytical conditions employed have been published previously (Landin *et al.*, 1996).

1.2.6 Calculation of quantitative values

The equation of linear regression (Y = aX + b) was calculated from the peak area ratios (Y) (d5-diHOPrVal / diHOPrVal) and mass ratios (X) (d5-diHOPrVal [mol] / diHOPrVal [mol]), that were obtained from the analysis of standard solutions. For sample analyses, mass ratios were obtained by assigning the peak area ratios of the samples to the above equation. Mass values of d5-diHOPrVal were then calculated by multiplying the mass ratios obtained by the diHOPrVal mass values (pmol). Finally, the quantitative values (pmol/g globin) were calculated by dividing the diHOPrVal mass value (pmol) by globin weight (g).

1.2.7 Analytical reproducibility and sensitivity

The GC-NCI-MS/MS chromatogram and mass spectrum of acetylated PFPTH-diHOPrVal are shown in Fig. 7 and Fig. 8. As reported by Landin *et al.* (1996), the derivative of acetylated PFPTH-diHOPrVal (M_r 482) gave a major fragment *m/z* 379 and its fragment ion *m/z* 337, which probably corresponded to $[M-103]^-$ ($M-HF-[CH_3]_2[CH]_2O$) and $[M-145]^-$ ($M-HF-[CH_3]_2[CH]_2O-CCH_3O$), respectively (Fig. 8 and Fig. 9).

In order to evaluate the reproducibility of analysis, diHOPrVal adduct levels in an exposed-rat globin sample were quantified by the modified Edman method, using GC-NCI-MS/MS. Between-run and within-run reproducibility of diHOPrVal adduct quantification were examined by analyzing the same sample three times on three different days (3 × 3 = 9 times). The coefficient of variation (CV, %) was determined by dividing the standard deviation by the mean value, and multiplying by 100. Analysis of variance (ANOVA) was performed to examine the between-run reproducibility, using

Microsoft Excel 2003; $p < 0.05$ was considered statistically significant. Regarding analytical sensitivity, a signal-to-noise ratio (S/N) of ten was used for estimating the lower limit of quantification (LOQ).

1.3 Results and discussion

Between-run and within-run reproducibility for the analysis of exposed-rat globin samples are shown in Fig. 10. Between-run CV was 5.5-12% (average 8.8%, n = 3 per analysis) and within-run CV was 12% (n = 9). No significant variance was observed between the levels determined in the same sample analyzed in different runs ($p > 0.05$). The LOQ was estimated to be 0.6 pmol/g globin, which was more sensitive than previously reported (Landin *et al.*, 1996). diHOPrVal adduct levels in globin samples from rats that were not exposed to G were measured in the same manner. The average diHOPrVal level of non-exposed rats was 7.2 ± 0.91 (average ± SD) pmol/g globin (Fig. 11), significantly lower than the level in exposed-rat samples (71.8 ± 6.5 pmol/g globin). The within-run CV was 5.8-15% (average 9.5%, n = 3 per analysis). These data suggested that diHOPrVal formation occurred *in vivo* in rats administered G, although the sample analyzed included Hb from exposed and non-exposed rats.

Based on these results, we concluded that a sensitive method for quantification of diHOPrVal in Hb had been established.

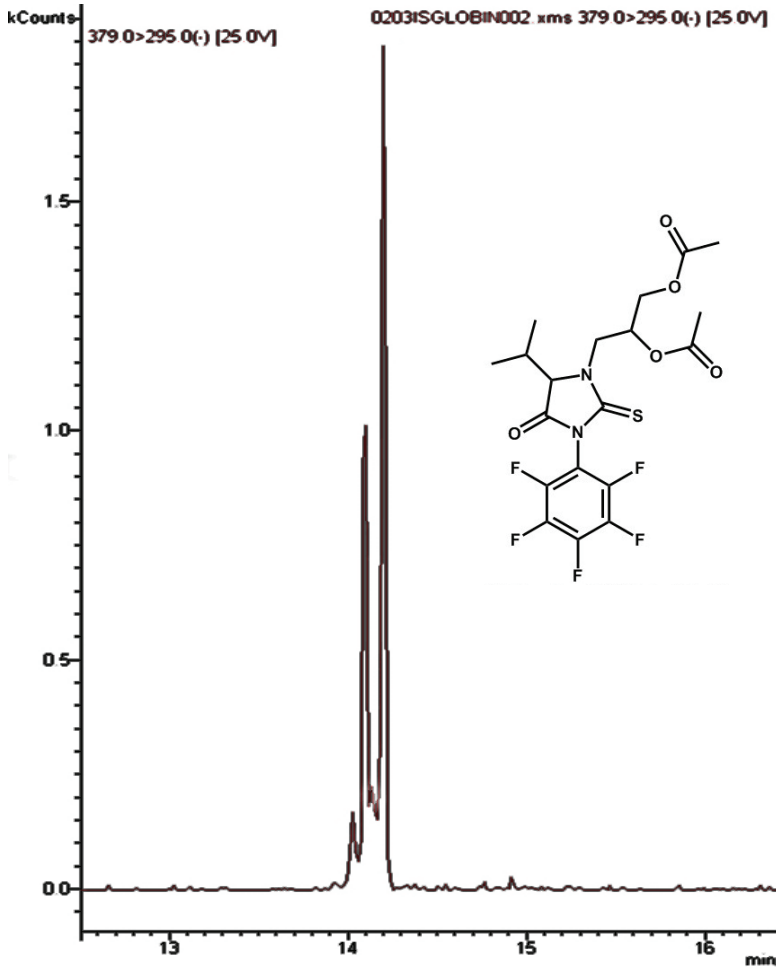

Fig. 7 GC-NCI-MS/MS chromatogram of acetylated PFPTH-diHOPrVal

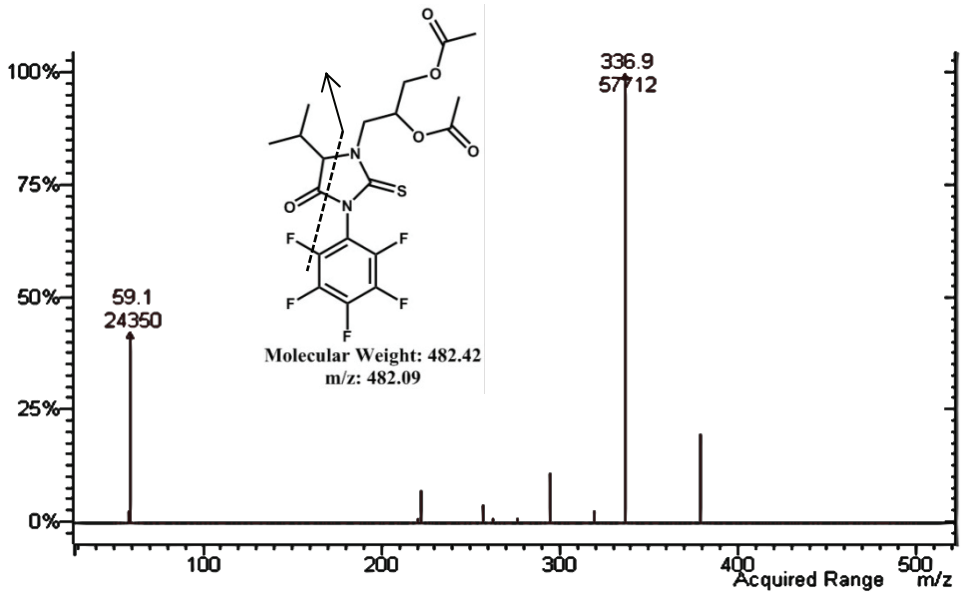

Fig. 8 NCI mass spectrum of acetylated PFPTH-diHOPrVal

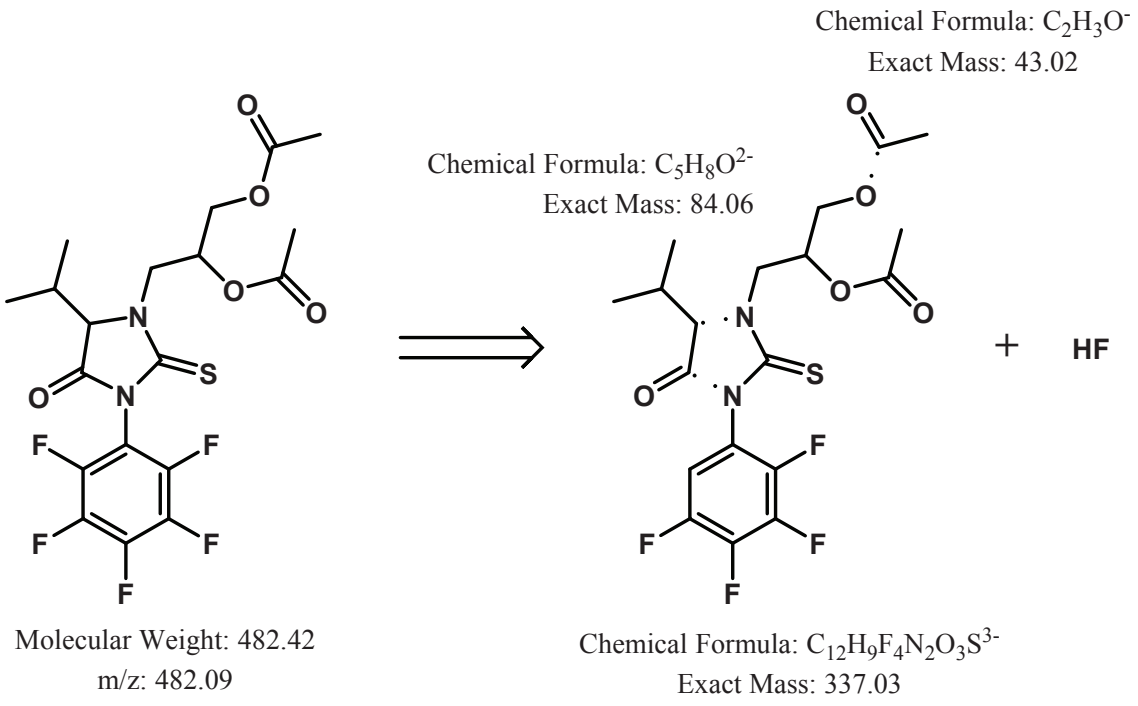

Fig. 9 Possible fragmentation of acetylated PFPTH-diHOPrVal in GC-MS/MS analysis

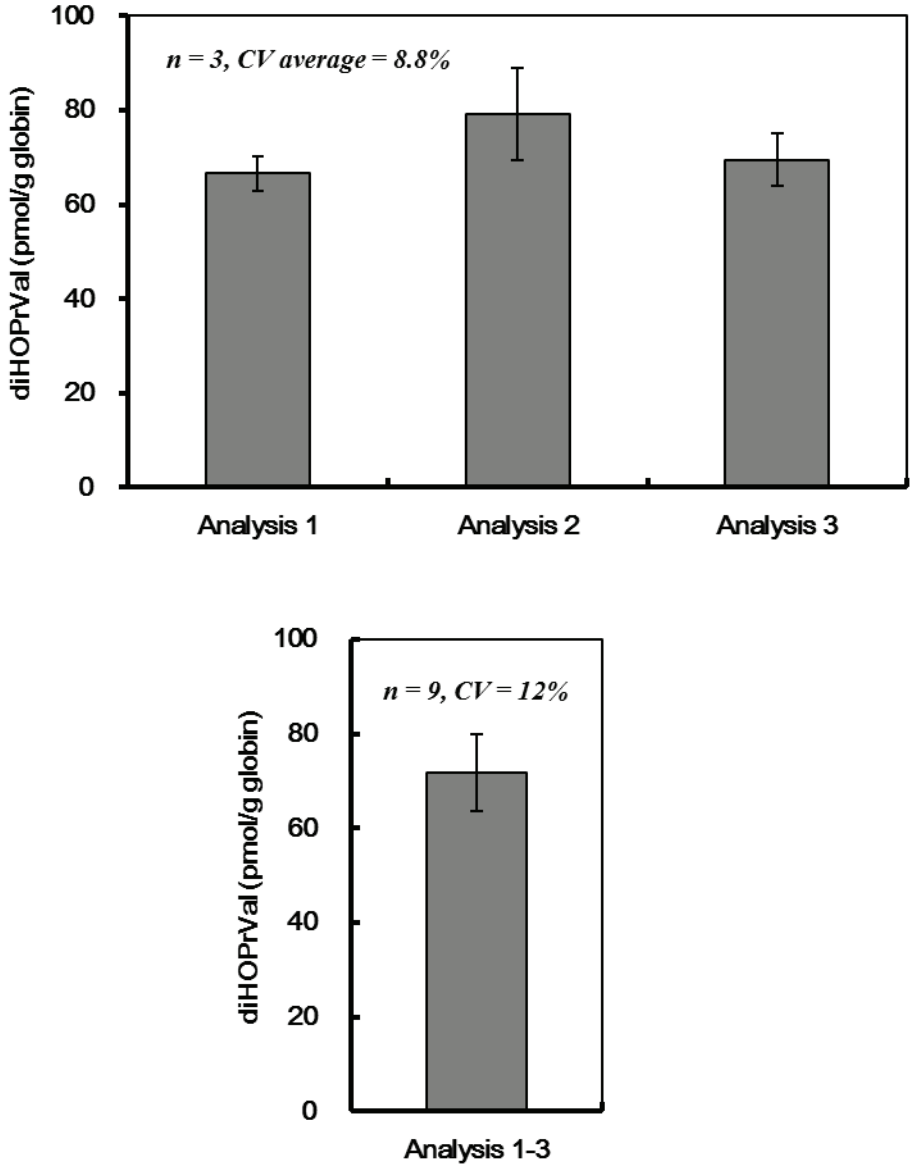

Fig. 10 Between-run and within-run reproducibility of diHOPrVal quantification

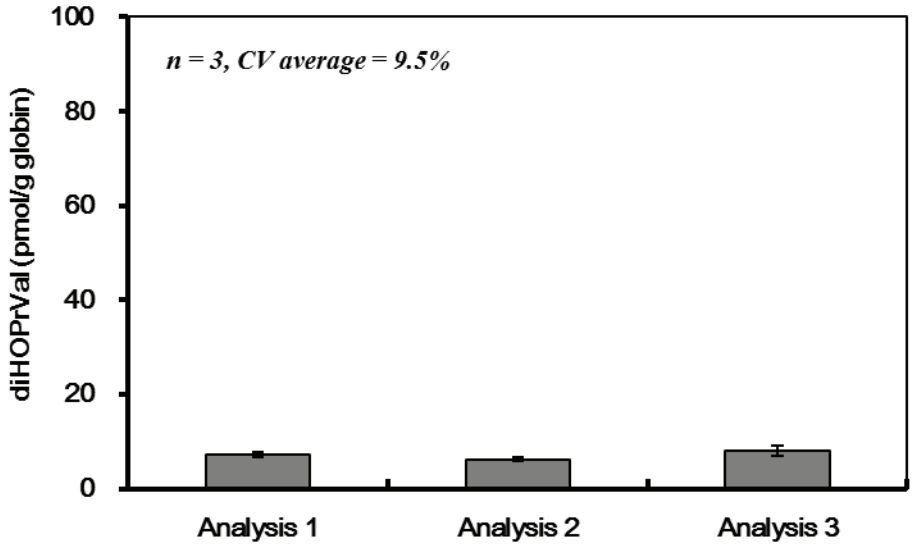

Fig. 11 Measurement of diHOPrVal adducts in rats that had not been exposed to G

Chapter 2. Characterization of the G-Hb adduct as an *in vivo* dose and exposure marker

2.1 Introduction

To investigate the usefulness of diHOPrVal G-Hb quantification, the G dose-dependency of the adduct and its ability to predict exposure, as well as its chemical stability *in vivo,* were determined. These results of these investigations were presented in Paper III (Honda *et al.*, 2014).

2.2 Materials and methods

2.2.1 Chemicals, analytical conditions and standards

The chemicals, analytical conditions, standards, method for globin sample preparation and derivatization were as described in 1.2.

2.2.2 *In vivo* formation and elimination of diHOPrVal in rats

2.2.2.1 Administration of G and collection of blood samples

Six-week-old male Sprague-Dawley rats were obtained from Charles River Laboratories Japan, Inc. (Yokohama, Japan) and kept on a 12-h light/dark cycle with free access to food and water. G was dissolved in distilled water and administered by gavage to 7-week-old rats in a volume of 5 mL/kg bw. Control animals were given vehicle only. The bw of each animal (271 g to 308 g) was within the mean bw ± 20%.

Whole blood was drawn from the abdominal aorta into a syringe under isoflurane anesthesia. Samples were collected using a syringe pretreated with heparin sodium anticoagulant (1, 000 IU/mL), and cooled on ice.

In order to characterize the relationship between administered dose and *in vivo* dose of G, groups of three rats were administered with G (0, 4.92, 12, 30, or 75 mg/kg bw) and blood was drawn 24 h after administration. The highest dose (75 mg/kg) was the highest dose included in the rat carcinogenicity test (NTP, 1990), and the lower doses were set with a common ratio of about 2.5. The highest dose used in the previous single oral dose toxicokinetic study in rats was 4.92 mg/kg bw (Wakabayashi *et al.*, 2012). Rat bw increased by 4 ± 3 g during this study.

We also analyzed the kinetics of diHOPrVal Hb adduct elimination from the circulation. Two groups of rats were administered 0 or 12 mg/kg bw of G. Blood was drawn 1 and 40 days after administration in the 0 mg/kg bw group and 1, 10, 20, and 40 days (n = 3 per group) after administration in the 12 mg/kg bw group. Rat bw increased by 5 ± 2 and 204 ± 54 g at 1 and 40 days, respectively, after administration in the 0 mg/kg bw group, and by 7 ± 2, 38 ± 62, 136 ± 26.5, and 205 ± 30.5 g at 1, 10, 20, and 40 days after administration, respectively, in the 12 mg/kg bw group.

2.2.2.2 Analysis of diHOPrVal elimination and determination of the first-order elimination rate constant

The theoretical background was described in the relevant section of the Introduction (Kinetics of Hb adduct formation and elimination, see equations 6 and 7). To determine diHOPrVal adduct stability *in vivo*, the decrease in initial adduct level (A_0) over time

was monitored in rats following a single exposure.

The k_{el} was optimized by mathematical modeling using Solver (Microsoft Office Excel 2003). The experimental data were compared with equations 6 and 7, where the unknown constants k_{el} and A_0 were optimized. The values of these variables that minimized the sum of the squares of the differences between the theoretical and experimental values were calculated.

2.2.2.3 Accumulation of adduct during chronic exposure

diHOPrVal was considered to be a generally occurring adduct in humans, because a certain background level of the adduct was observed in unexposed control individuals (Paper II [Honda *et al.*, 2012]; Landin *et al.*, 1997). In order to estimate the *in vivo* dose of G assumed to cause the background levels of diHOPrVal adducts observed in humans, adduct accumulation has to be considered. The theoretical background was described in the relevant part of the Introduction (Kinetics of Hb adduct formation and elimination, see equations 1, 2b, 3, 4, and 5).

2.2.3 *In vitro* determination of the second-order reaction rate constants for G binding to the Hb *N*-terminal valine

2.2.3.1 Blood sample collection

Human blood samples were obtained from non-smoking volunteers, who were employees of Kao Corporation. The study complied with the regulations of the company ethical committee, and included obtaining written informed consent and providing

written information explaining the objectives of the study to the donors in advance. Blood samples were collected from the donors using heparinized vacutainer tubes. Rat blood samples were collected as described in section 2.2.2.1. The donors and the rats were fasted for 12 h before blood samples were taken.

2.2.3.2 Erythrocyte incubation with G and calculation of the second-order reaction rate constant

Three whole-blood samples from a total of 9 rats (each sample was pooled from 3 rats) and whole blood samples from 3 donors were incubated with different concentrations of G (0, 50, 100, 200, and 400 µM) at 37°C for 1 h to measure the second-order reaction rate constant (k_{val}) of G with the Hb *N*-terminal valine. Aqueous solutions (40 µL) of G were added to whole blood samples (final volume, 4 mL) in polypropylene tubes. Reactions were terminated by addition of 5 volumes of ice-cold saline solution, quickly followed by centrifugation and removal of the plasma. Erythrocytes were then washed twice prior to globin sample preparation as described in section 1.2.4, and diHOPrVal levels were measured. The concentrations of G were assumed to be constant during the short incubation time (1 h) (Paulsson *et al.*, 2005).

As indicated by equations 2a and 2b (stated previously), and 2 c (below), the k_{val} (expressed as pmol/g globin per µMh) for the formation of Hb adducts can be calculated from the Hb adduct level measured after *in vitro* treatment of blood with known concentrations of G for short incubation times,

$$k_{val} = A / D \qquad (2c)$$

where A is the measured adduct level (pmol/g globin), D is the AUC or *in vitro* dose (μMh = μM × h = μmol/L × h). The dose (D) is estimated from the concentration of G at the beginning of the experiment (C) and exposure time (t), as described in equation 1.

2.2.4 Statistics

The dose dependency and elimination of diHOPrVal were analyzed by multiple comparison tests. Firstly, the data were analyzed for homogeneity of variance using Bartlett's test. When the variances were homogeneous, the Williams test was used, and when the variances were heterogeneous, the Shirley-Williams test was performed. Welch's t-test (two-tail) was used to compare the human and rat second-order reaction rate constants (k_{val}). All statistical analyses were conducted using Microsoft Excel 2003. A level of $p < 0.05$ was considered statistically significant.

2.2.5 Quality control

One globin sample with a known diHOPrVal level was included in every run of approximately 8 samples, for quality control (QC) purposes. If the diHOPrVal level of the QC sample was out of the range of the mean concentration, all the samples in that run were re-analyzed.

2.3 Results and discussion

2.3.1 Dose-related adduct formation *in vivo*

Rats were administered G at different doses (0, 4.92, 12, 30, and 75 mg/kg bw), and the diHOPrVal Hb adduct levels in the blood were measured 24 h later (Fig. 12). diHOPrVal levels increased significantly in rats exposed to G, compared to those of the control group. The extent of the increase depended on the dose administered (Shirley-Williams test, $p < 0.05$ at 4.92 mg/kg bw and $p < 0.01$ at 12 mg/kg bw or more). Linear regression analysis showed a high positive correlation between G dosage and diHOPrVal levels (coefficient of determination [R^2] = 0.943).

Several previous animal experiments demonstrated that G was readily bioavailable after oral administration (Nomeir *et al.*, 1995; Wakabayashi *et al.*, 2012). In the present study (Paper III [Honda *et al.*, 2014]), the strong linear correlation between the dose of G and diHOPrVal levels 24 h after oral administration to rats indicated that G was rapidly absorbed and bound to Hb in a dose-dependent manner. Models that take erythrocyte lifetime into account have been published for adduct accumulation and elimination kinetics, and these have been adapted to model various exposure scenarios, including a single exposure (Fennell *et al.*, 1992; Granath *et al.*, 1992). The relationship between the measured steady-state adduct level and the daily increment in Hb adduct level during chronic exposure was described by equation 4, whilst equation 5 described the relationship between *iDEL* and the daily increment in Hb adduct level. Continuous G exposure resulted in accumulation of diHOPrVal over the erythrocyte lifetime (equation 4). Linear regression analysis of the present rat *in vivo* study data (Fig. 12) indicated that the unit diHOPrVal increase was approximately 55 pmol/g globin per mg G/kg bw in rats. Using equation 4, if A_d is equivalent to the adduct levels formed after administration of a single dose, this value would be 1.7 nmol/g globin per mg G/kg bw during chronic exposure to equivalent daily doses ($t > t_{er}$). In other words, daily

exposure to 0.60 μg G/kg bw would result in a 1 pmol diHOPrVal/g globin increase in the steady-state level (A_{ss}). Since the lifetime of human erythrocytes (123 days, Furne *et al.*, 2003) is about double that of rat erythrocytes (61 days, Derelanko *et al.*, 1987), more sensitive monitoring of *in vivo* G exposure would be expected in humans, because of the longer time period for accumulation of Hb adducts, assuming that there are no major species differences in the kinetics of diHOPrVal formation.

2.3.2 Chemical stability *in vivo*

Four groups of rats (n = 3 per group) were administered a single dose of G (12 mg/kg bw) and blood samples were collected 1, 10, 20, and 40 days after administration (Fig. 13, upper curve). diHOPrVal levels decreased linearly in a time-dependent manner (Williams test, $p < 0.01$ at 40 days), consistent with the normal turnover of erythrocytes. The first-order elimination rate constant (k_{el}) was calculated using equation 6, optimized with Solver. The k_{el} was 0.00011, suggesting that the adducts were chemically stable, although the coefficient of determination was lower ($R^2 = 0.717$). Since the k_{el} determined using equation 7 gave a negative value, we did not apply it. Accordingly, k_{el} contained a blood dilution effect resulting from the bw gain in this experiment.

The elimination of diHOPrVal in rats following a single administration was time-dependent, indicating zero-order elimination behavior (k_{el} = 0.00011). The diHOPrVal adducts appeared to be stable over the lifetime of the erythrocytes, as expected. Meanwhile, an example of an unstable adduct with elimination kinetics that did not show zero-order behavior but were compatible with first-order behavior, has been reported (Fennell *et al.*, 1992; Granath *et al.*, 1992). Fig. 13 illustrates a

simulation of the elimination of stable (ethylnitrosourea: ENU); k_{el} = 0.0023 (Bergmark *et al.*, 1990) and unstable (4,4′-methylenebis (2-chloroaniline): MOCA); k_{el} = 0.2771 (Cheever *et al.*, 1990; Fennell *et al.*, 1992) Hb adducts, based on equation 6. The simulation curve for diHOPrVal showed better agreement with that of the ENU Hb adduct, than with that of the MOCA Hb adduct. Therefore, once formed, diHOPrVal Hb adducts were considered stable over the lifetime of the erythrocyte. Landin *et al.* (1999) also reported formation and elimination of diHOPrVal in rats following administration of an acute intraperitoneal dose of epichlorohydrin. In their study, diHOPrVal levels showed delayed formation but sustained high levels for 10–20 days. The diHOPrVal adduct in Hb is formed from epichlorohydrin in a second reaction, following the initial formation of N-(3-chloro-2-hydroxypropyl)valine adducts (Bader *et al.*, 2009). The kinetics of diHOPrVal formation from G were not characterized in the epichlorohydrin exposure study. In the present study, the formation of diHOPrVal in Hb directly from G and the *in vivo* stability of the diHOPrVal adducts were characterized.

2.3.3 Determination of the second order reaction rate constant

To investigate the reactivity of G with the Hb *N*-terminal valine, samples of rat and human whole blood were incubated with G at different concentrations (0, 50, 100, 200, and 400 μM) for 1 h at 37°C. A concentration-dependent formation of diHOPrVal was observed in these samples, with R^2 = 0.88–0.94 in rats (Fig. 14) and R^2 = 0.91–0.92 in humans (Fig. 15). The second-order reaction rate constants (k_{val}) were calculated for both species using equation 2c, as shown below:

k_{val}-rat = 6.7 ± 1.1 pmol/g per µMh

k_{val}-human = 5.6 ± 1.3 pmol/g per µMh

There was no significant species difference in G reactivity with the *N*-terminal valine of rat and human Hb (p = 0.293). The curves from the *in vitro* k_{val} determinations indicated a somewhat lower adduct level increment per dose unit at lower concentrations of G, probably because the concentration was somewhat reduced during the 1-h incubation, but the calculations assumed that it was constant. This reduction in G concentration may occur due to reaction with glutathione, and would have less impact on the data generated in the presence of higher concentrations. This emphasizes the importance of studying the effects of several concentrations of the electrophile in these types of experiments. In a similar study of ethylene oxide and glycidamide, no similar trend towards lower increases in adduct level per dose unit at lower concentrations was observed using a short incubation period with whole blood (Paulsson *et al.*, 2005). The reactivity of G with different model nucleophiles has been measured, indicating that reactivity (measured as an s value) was close to that of ethylene oxide and glycidamide (Ohlén, A., 2005; Silvari *et al.*, 2005), as expected. The second-order reaction rate constants towards the *N*-terminal valine of human Hb were 62 pmol/g globin per µMh for ethylene oxide (Granath *et al.*, 1999) and 21 pmol/g globin per µMh for glycidamide (Vikström *et al.*, 2011). Some measured k_{val} values of other low molecular weight electrophiles (Table 1) suggested similar reactivity towards the Hb *N*-terminal valine as that of G.

2.3.4 Prediction of *in vivo* dose

The k_{val} values were used for calculation of the *in vivo* dose in rats administered 4.92 and 75 mg G/kg bw using the diHOPrVal levels. A previous rat toxicokinetic study of single oral doses of G included the above doses (Wakabayashi *et al.*, 2012). The *in vivo* doses estimated in the present study (Paper III [Honda *et al.*, 2014]) were compared with the corresponding AUC values measured in the plasma in this previous toxicokinetic study, which was conducted under the same conditions as the present study but involved the use of different animals (Fig. 16). There was good agreement between the AUC values and the *in vivo* doses obtained using these two methods at both doses (4.92 and 75 mg/kg bw). Hence, the measurement of diHOPrVal was useful, not only for proving and/or for comparing G exposure, but also for quantitatively assessing the *in vivo* dose (AUC in blood). Similar attempts to estimate the *in vivo* dose from Hb adduct levels have been reported for acrylamide and glycidamide (Törnqvist *et al.*, 2008; Vikström *et al.*, 2011) and for several other simple alkylating agents. Indeed, the original application of *in vivo* Hb adduct level measurement was for AUC estimation (Ehrenberg *et al.*, 1983). However, to the best of our knowledge, no previous reports have described the estimation of AUC using the relationship between *in vitro* k_{val} and *in vivo* Hb adduct levels, with subsequent comparison with measured *in vivo* AUC values. Tareke *et al.* (2006) indirectly discussed the relevance of k_{val} derived from *in vitro* experiments by comparing it with "k_{val} *in vivo*", obtained from measurement of acrylamide and glycidamide *in vivo* Hb adduct levels and AUC. Using ethyl methanesulfonate, Lavé *et al.* (2009) generated data that could be used for comparison of the measured plasma AUC with the *in vivo* dose, calculated from Hb adduct levels. However, the above studies did not determine the consistency between the k_{val}

determined *in vivo* and *in vitro,* or between doses. The present study (Paper III [Honda *et al.*, 2014]) determined k_{val} *in vitro,* taking the experimental conditions into consideration (e.g., use of whole blood and incorporation of deviations in the estimation of k_{val}, etc.), and showed that k_{val} could be used to estimate the *in vivo* G dose (AUC) accurately based on diHOPrVal levels. Measuring Hb adducts after determination of k_{val} is thus a valuable tool for the measurement of daily exposure levels to reactive chemicals *in vivo*.

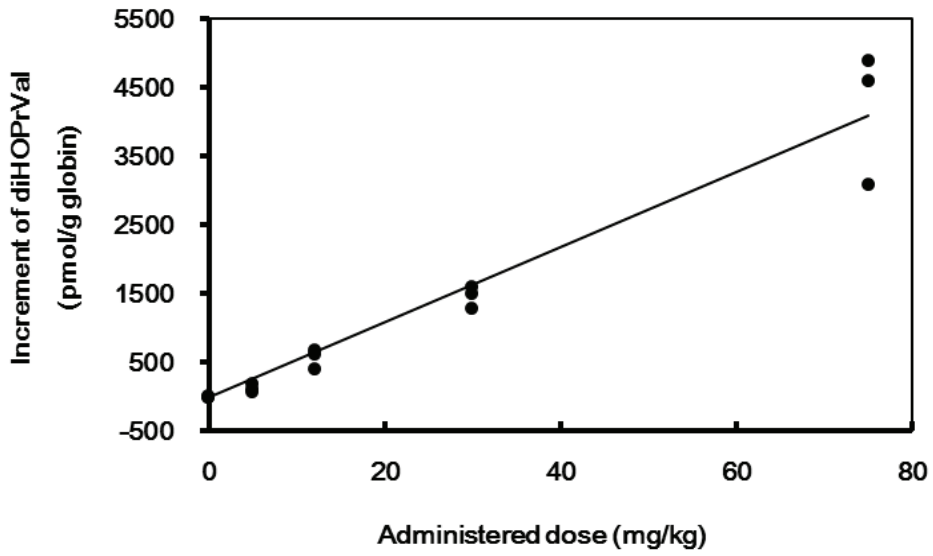

Fig. 12 Formation of diHOPrVal in rats administered oral G

The y-axis begins from a negative value because the background diHOPrVal level was subtracted from each data point

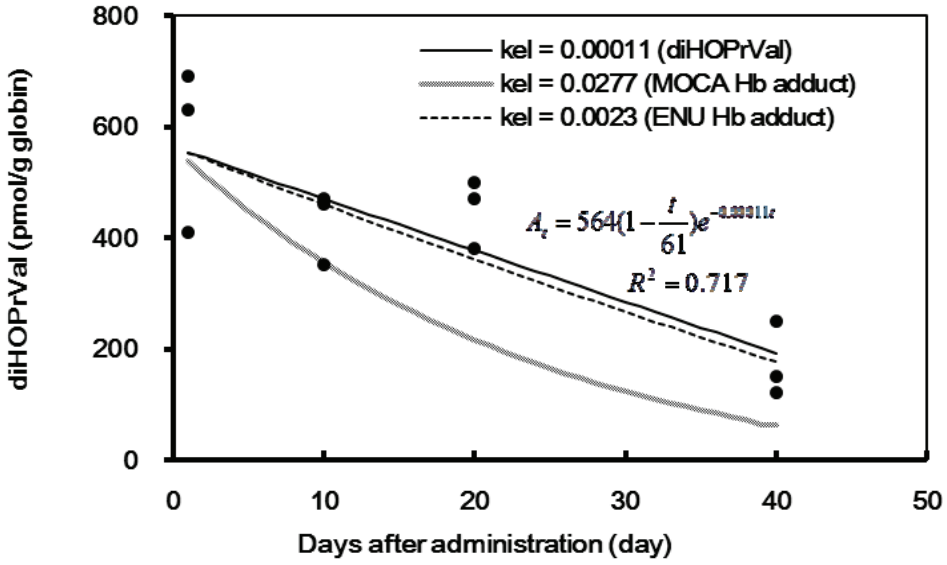

Fig. 13 Elimination of diHOPrVal in rats administered a single oral dose of 12 mg G/kg bw.

Elimination of the stable ethylnitrosourea (ENU) Hb adduct and unstable 4,4′-methylenebis (2-chloroaniline) (MOCA) Hb adduct is shown for comparison (from Bergmark *et al.* (1990) and Cheever *et al.* (1990), and Fennell *et al.* (1992), respectively).

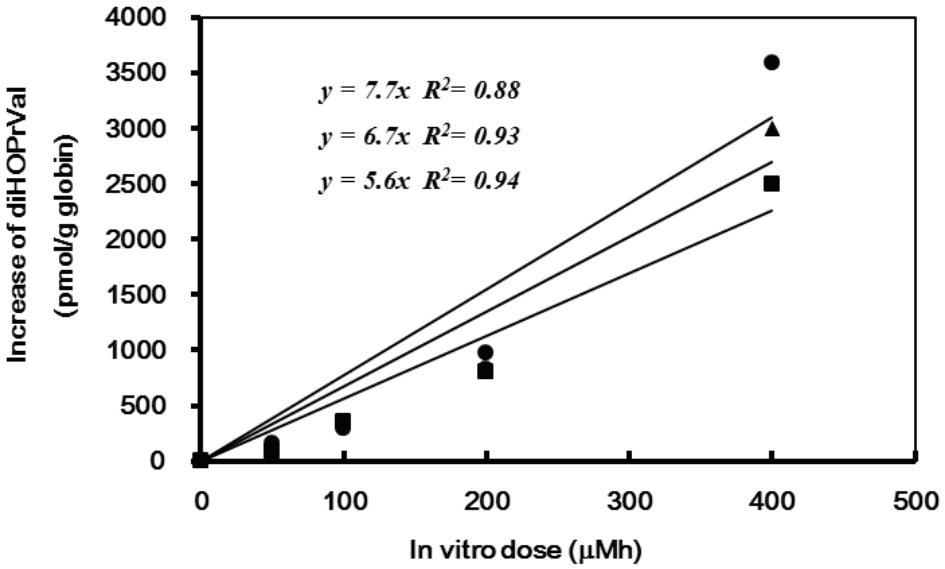

Fig. 14 Determination of the *in vitro* second-order reaction rate constant ($k_{val\text{-}rat}$) for the reaction between G and the Hb *N*-terminal valine.

The y-axis begins from zero because the control level (0 μMh) was subtracted from each data point. Circles, triangles, and squares indicate data from each sample.

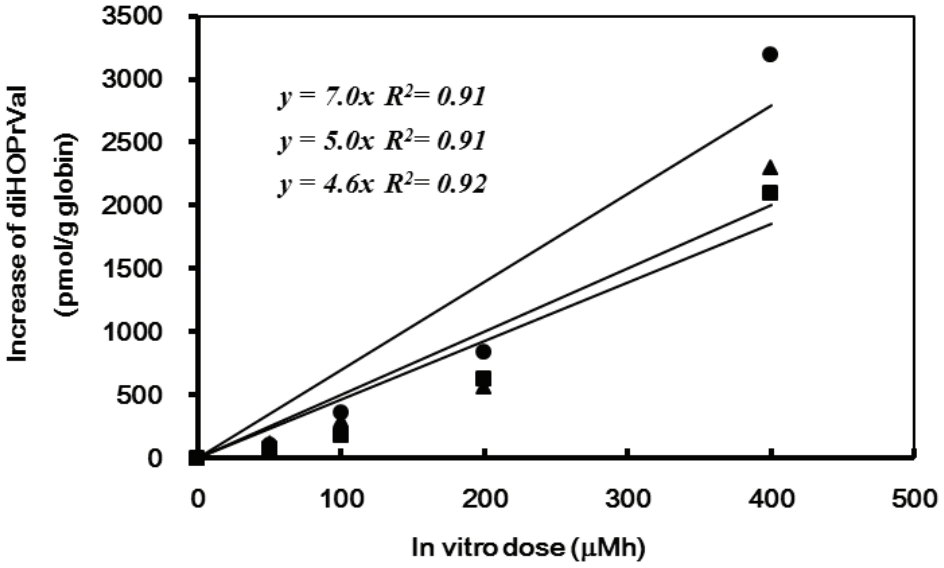

Fig. 15 Determination of the *in vitro* second-order reaction rate constant ($k_{val\text{-}human}$) for the reaction between G and the Hb *N*-terminal valine in human Hb.

The y-axis begins from zero because the control level (0 μMh) was subtracted from each data point. Circles, triangles, and squares indicate individual data.

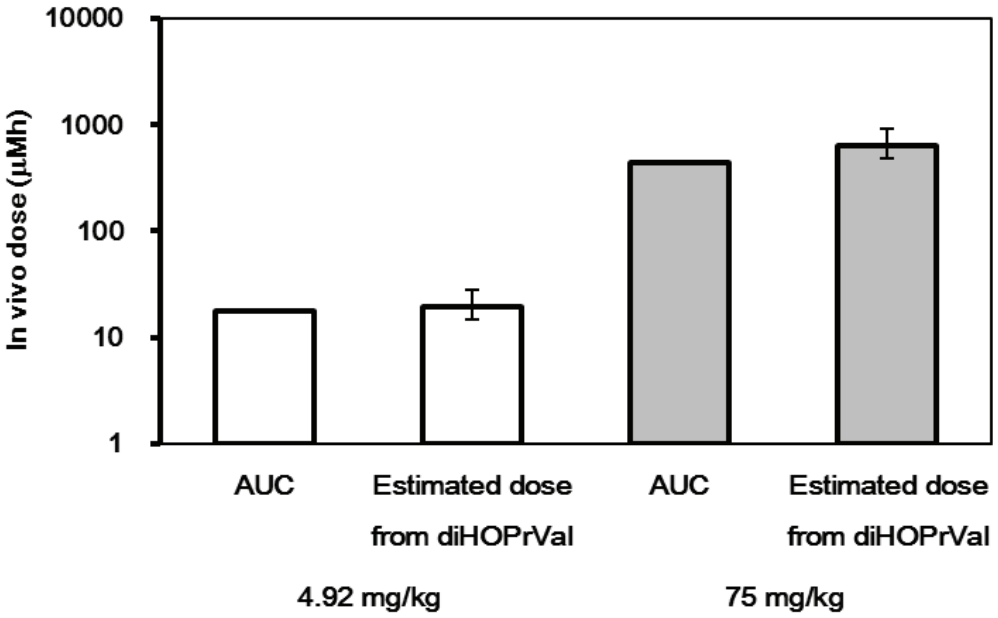

Fig. 16 Comparison of the *in vivo* dose estimated from the diHOPrVal levels, and the AUC determined in a rat toxicokinetics study (Wakabayashi *et al.*, 2012)

The white and gray bars show the AUC and estimated *in vivo* doses in rats administered oral G at 4.92 and 75 mg/kg bw, respectively. Error bars indicate the range of estimated doses calculated from the variation of k_{val} (mean ± 2SD).

Table 1 Second-order reaction rate constants for the reactions of the indicated chemicals with the *N*-terminal valine (k_{val}) of Hb

Reference	Second-order reaction rate constant (pmol/g globin per μMh)	
	Human	Rat
$k_{val\text{-}glycidol}$		
Present study	5.6 ± 1.1	6.7 ± 1.3
$k_{val\text{-}acrylamide}$		
Vikström *et al.* 2011	6.4	nd
Törnqvist *et al.* 2008	nd	4.6
Fennell *et al.* 2005	4.3	3.8
Tareke *et al.* 2006	7.4	2.9
$k_{val\text{-}glycidamide}$		
Vikström *et al.* 2011	21	nd
Törnqvist *et al.* 2008	nd	14
Fennell *et al.* 2005	6.7	5.0
Tareke *et al.* 2006	59	9.5
$k_{val\text{-}propylene\ oxide}$		
Segerbäck *et al.* 1994	25	22
Pauwels *et al.* 1998	16*	nd
$k_{val\text{-}ethylene\ oxide}$		
Pauwels *et al.* 1998	42*	nd
Granath *et al.* 1999	62	58

* These values were calculated based on Hb's molecular weight of 64,000.

Chapter 3. G exposure evaluation in humans who ingested DAG oil containing GEs

3.1 Introduction

Measurement of Hb adducts was used to evaluate G exposure in humans who had ingested DAG oil containing GEs. We previously measured diHOPrVal levels in 7 DAG oil-exposed donors and 6 non-exposed donors and found no significant difference between them (Paper I [Honda *et al.*, 2011]). In fact, Hb adduct levels were lower in DAG oil users (3.5 ± 1.9 pmol/g globin), compared to non-users (7.1 ± 3.1 pmol/g globin). However, the report of a similar difference between Swedish and German subjects without any known chemical exposure (Landin *et al.*, 1997) indicated that it would be better to consider a larger study sample in order to analyze the statistical significance of this difference. We therefore conducted an additional analysis of DAG oil-exposed subjects and non-exposed volunteers (Paper II [Honda *et al.*, 2012]). Since most of samples from Paper I (Honda *et al.*, 2011) (11 non-smoker samples) were incorporated into Paper II (Honda *et al.*, 2012), the details of Paper I were omitted.

3.2 Materials and methods

3.2.1 Chemicals, analytical conditions and standards

The chemicals, analytical conditions, and standards were as described in 1.2.

3.2.2 Information regarding donors

All human blood samples were obtained from volunteer employees of the Kao Corporation and the following procedures were regulated and approved by the company ethical committee. Blood samples were obtained with written informed consent of the donors, following an explanation of the study objectives. Even after the company halted marketing of DAG oil, many consumers (including company employees) voluntarily continued to use the remaining DAG oil for their food preparation at home. Furthermore, food prepared using DAG oil was served in the company cafeteria for a few months, before stocks were exhausted. DAG oil-exposed donors were defined as individuals who had used the cafeteria for lunch daily (over 20 times/month) and who also used DAG oil at home for cooking. Non-exposed donors were defined as individuals who had not used DAG oil in the 4 month period before the blood samples were provided.

3.2.3 Matched case-control study

As described in Paper I (Honda *et al.*, 2011), we previously analyzed blood samples from 7 DAG oil-exposed donors and 6 non-exposed donors, a total of 13 samples. We identified an additional 8 DAG oil-exposed donors for analysis in Paper II (Honda *et al.*, 2012). One DAG user who smoked was excluded from this study. A total of 14 DAG oil-exposed donors were therefore evaluated. The previous study only recruited 6 non-exposed donors, because most of the employees had eaten foods prepared using DAG oil in the cafeteria. After the cafeteria stopped using DAG oil, 42 non-exposed donors were identified with a sufficient interval of non-use (more than the 4-month life

span of the human erythrocyte).

As this study was designed as a retrospective matched case-control study, three controls (non-exposed donors) were matched with each DAG oil-exposed donor using the variables of gender and age (± 3 years). As mentioned above, no smokers were enrolled in the study.

3.2.4 Discontinuation of DAG oil usage

Hb adduct levels were measured initially in 21 donors, and measured again in 17 of these donors (12 DAG oil-exposed and 5 non-exposed) at a sufficient time interval after cessation of DAG oil ingestion (more than 4 months). The remaining 4 donors were not suitable for re-evaluation because of technical or personal reasons, for example discontinuation of DAG oil use at home.

3.2.5 Risk assessment

The MOE and iMOE approaches were employed for carcinogen risk assessment, as explained in the background section relating to carcinogen risk assessment. Firstly, MOE was applied to evaluate the risk associated with DAG oil ingestion, as compared to the risk associated with daily acrylamide exposure. Secondly, exposed and non-exposed subject risk was evaluated using iMOE. The theoretical background was described in the relevant section of the Carcinogen risk assessment background (Margin of exposure and Internal MOE, see equations 8, 9 and 10).

3.2.6 Analysis of donor diet history

All donors were asked to complete a brief self-administered diet history questionnaire (BDHQ) (Sasaki, 2004) to provide information about their dietary habits. The BDHQ included 70 food items and 97 nutrients, and assessed dietary history over the previous month. The daily ingestion of edible oil was calculated using the BDHQ. This did not necessarily mean that only DAG oil was consumed by the DAG oil-exposed group, or that only triacylglycerol (TAG) oil was consumed by the non-exposed group. Nevertheless, DAG oil-exposed donors were considered to use mainly DAG oil, and non-exposed donors were considered to use TAG oil as their major edible oil prior to blood sampling. Since smoking was indicated to affect diHOPrVal formation (Landin *et al.*, 1997), information regarding smoking habits was also obtained.

Using the BDHQ data, we attempted to identify food items that were ingested in larger amounts by donors with relatively high (above the median) diHOPrVal levels (high exposure group), compared to those with below-median diHOPrVal levels (low exposure group). For this analysis, we calculated the ratio of the daily intake in these groups (high exposure group / low exposure group) and the mean difference in daily intake was analyzed (Welch's t-test). In addition, the correlation between the daily intake of each food and the diHOPrVal level was calculated (Pearson's correlation coefficient). Finally, the foods and food components that showed a significant correlation with diHOPrVal adduct levels were selected. These had a daily ingestion ratio of > 1.25 or < 0.8, Welch's t-test p-values of < 0.1, and Pearson's correlation coefficients of > 0.2 or < -0.2). All statistical analyses were conducted in Microsoft Excel 2003.

3.2.7 Statistics

Two-way ANOVA was performed for the matched case-control pairs using Statistical Analysis Software (SAS, version 9.2, SAS Institute Inc., Cary, NC, USA). A paired t-test (one-tail) was used to compare donor diHOPrVal levels before and after discontinuing DAG oil usage. Correlations between diHOPrVal levels and estimated food consumption or other variables (age, body weight, and gender) were analyzed by Spearman's correlation. Grubbs' test was applied to remove outliers. When the determined adduct value was considered to be outside the range of non-exposed donors in the previous study (mean ± 3SD) (Paper I [Honda *et al.*, 2011]), we analyzed the same sample three more times (4 analyses in total) and judged whether the value was an outlier or not. If the value was judged to be an outlier, it was removed and Grubbs' test was repeated. Otherwise, the average value of the 4 analyses was adopted as the definitive value. All statistical analyses, except for two-way ANOVAs, were conducted in Microsoft Excel 2003; $p < 0.01$ was considered significant in the Grubbs' test, and $p < 0.05$ was considered significant in the other analyses.

3.3 Results

3.3.1 Matched case-control study

In order to increase the credibility and relevance of the evidence, a matched case-control study was performed. The study design allowed donors to be grouped according to factors such as age and gender differences, to reduce the variability introduced by these factors. Comparisons of user outcomes were made within each group, and were integrated across groups. As the marketing of DAG oil was halted in

2009, we only secured 14 samples in total from DAG oil-exposed donors, including 7 previously-evaluated samples. We obtained 42 samples from non-exposed donors for the matched case-control study, and therefore allocated 3 controls to each case (DAG oil-exposed subject), matching their age and gender.

Individual diHOPrVal levels and relevant donor information are shown in Table 2, and results of the two-way ANOVA analysis are presented in Table 3. G-Hb adducts were quantified in all samples. No significant case vs. control difference in the diHOPrVal level was observed (ANOVA). The average level of diHOPrVal in the DAG oil-exposed group was 7.3 pmol/g globin (95% CI: 4.9–9.0), compared with 6.9 pmol/g globin (95% CI: 6.1–8.5) in the non-exposed group ($p = 0.744$). The 95% CI (-2.8–2.0) for the mean difference between matched case-control pairs across zero indicated that there were no significant differences between diHOPrVal levels in the DAG oil-exposed and non-exposed subjects. In addition, no significant difference was identified regardless of effect of grouping (one-way ANOVA, $p = 0.758$). Fig. 17 illustrates the distribution of individual diHOPrVal levels (A) and a box plot showing the median Hb adduct levels, with the 10, 25, 75, and 90th percentiles presented as vertical boxes and error bars (B). Median levels of adducts were approximated to average levels, and were positioned close to the center of each box, indicating that the distribution was close to normal for both groups.

3.3.2 Effect of discontinuing the use of DAG oil

Since DAG oil is not currently marketed and the company cafeteria stopped serving food prepared with DAG oil at the beginning of 2010, most of the

previously-investigated DAG oil-exposed donors no longer ingested DAG oil. We therefore re-evaluated diHOPrVal levels in these subjects at a sufficient interval after cessation (>4 months, the human erythrocyte life span), to examine the effect of discontinuing the use of DAG oil (Paper II [Honda *et al.*, 2012]).

Table 4 shows individual diHOPrVal levels and relevant donor information for blood samples obtained in the period before and after DAG oil-exposed donors discontinued DAG oil usage. For comparison, blood samples were also obtained from non-exposed subjects at the same time points. At the 2009-2010 time point, DAG oil-exposed donors used DAG oil, whereas in 2011, DAG oil was no longer available. Fig. 18 illustrates the change in individual diHOPrVal levels within the DAG oil-exposed group (A), and the distribution of Hb adducts is expressed as a box plot (B). The levels of diHOPrVal in the DAG oil-exposed group before and after discontinuing their use of DAG oil were 7.1 ± 1.1 (mean ± SD) and 7.5 ± 1.4 pmol/g globin, respectively, which did not represent a significant difference ($p = 0.403$). A similar difference in diHOPrVal levels was found in the non-exposed group in 2009-2010 (7.6 ± 3.1 pmol/g), compared with 2011 (9.7 ± 7.3 pmol/g).

3.3.3 Risk assessment

3.3.3.1 MOE approach

Acrylamide, a known animal carcinogen, has been reported to form *N*-(2-carbamoylethyl)valine (CEV) Hb adducts (EU, 2002; Fennell *et al.*, 2005; US EPA, 2010). In studies of occupational exposure to acrylamide, a conspicuous CEV adduct background level was observed in unexposed control individuals (Bergmark, 1997;

Hagmar *et al.*, 2001). Further studies to address the origin of this Hb adduct revealed that high levels of acrylamide were formed during the frying or baking of a variety of foods (Tareke *et al.*, 2002; Rosén and Hellenäs, 2002; US EPA, 2010). From the viewpoint of cancer risk assessment, the JECFA identified an MOE of 310, by comparing the average intake of acrylamide by the general population (0.001 mg/kg bw per day) with the $BMDL_{10}$ of 0.31 mg/kg bw per day for induction of mammary tumors in rats (JECFA 64th, 2004; JECFA 72nd, 2010). Similar MOE values can be calculated for the GEs found in DAG oil, based on the worst-case scenario that G is fully released during GE digestion. Assuming ingestion of 10 g DAG oil containing GEs at a concentration of 269 μg/g (Masukawa *et al.*, 2010) per day, an MOE of 342 can be calculated by comparing this human exposure (0.012 mg/kg bw per day) with the $BMDL_{10}$ for G (4.06 mg/kg bw per day, reported by BfR (2009a,b)) for induction of tunica vaginalis mesothelioma in rats (Paper I [Honda *et al.*, 2011]).

3.3.3.2 iMOE approach

The cancer risk derived from daily exposure to G-related compounds in humans without other known chemical exposures was discussed in Paper III (Honda *et al.*, 2014). We (Papers I and II [Honda *et al.*, 2011; 2012]) and Landin *et al.* (1997) reported similar average levels (2.1–7.3 pmol/g globin) of diHOPrVal in the blood of non-smokers without known chemical exposure. This indicated that there might be an unidentified source of daily G exposure. Assuming that G is the only cause of diHOPrVal adduct formation, and using the k_{val} determined in the Paper III (Honda *et al.*, 2014), we could estimate the *in vivo* dose of this unknown G source. Based on our reported average diHOPrVal

level of 7.3 pmol/g globin for non-smokers (Paper II [Honda *et al.*, 2012]), the daily increment of diHOPrVal was calculated to be 0.12 pmol/g globin (equation 4), corresponding to an *iDEL* of 22 nMh/day (equations 5 or 10). Consequently, iMOE was calculated to be 526 (equation 9). Taking together the *iDEL* for non-exposed humans (22 nM/day), and the relationship between oral dose and AUC in rats and monkeys administered G at 4.92 mg/kg bw (Wakabayashi *et al.*, 2012), these findings indicated that the oral G exposure range was 0.0061 to 0.027 mg/kg bw/day.

3.4 Discussion

3.4.1 Effect of DAG oil ingestion on internal exposure to G

The present study (Paper II [Honda *et al.*, 2012]) showed that there was no significant difference between the G-Hb adduct level in the DAG oil-exposed and non-exposed groups under these study conditions, using age- and gender-matched subjects and an increased sample size. The results of the two-way ANOVA indicated that age and gender did not significantly affect diHOPrVal levels ($p = 0.152$; Table 3). No correlation for these variables was confirmed by the Spearman's correlation. The present study indicated that there was either no increase in diHOPrVal levels, or minimal G exposure, in humans who ingested DAG oil containing small amounts of GEs. In addition, the average diHOPrVal levels in DAG oil-exposed donors did not significantly change after discontinuation of DAG oil use. This result further supported the finding that DAG oil ingestion did not increase G exposure in humans. Although notable changes in diHOPrVal levels were observed for several DAG oil-exposed donors (E7, E9, and E12 in Table 4), similar fluctuations were also seen for non-exposed donors (C1, C2, and C5 in

Table 4). These large changes could not therefore be attributed to the level of DAG oil ingestion.

Meanwhile, as described in 3.2.2, all samples were obtained from volunteer employees of Kao Corporation and the number of study subjects was relatively limited. These provided valuable data but as the subjects were recruited from a population with a similar working environment and lifestyle, the sample may be biased and unrepresentative of all DAG oil users. Although it would be ideal to study a larger, population-based sample set, this was impractical because DAG oil had been withdrawn from the market. Nevertheless, as described in section 3.2.2, employees of Kao Corporation are considered to be a population with high exposure to DAG oil and can therefore provide valuable data.

Our objective in measuring G-Hb adduct levels was to investigate whether the release of G during digestion of the GEs present in edible oil posed a risk to human safety. From the viewpoint of risk assessment, the present study (Paper II [Honda *et al.*, 2012]) indicated that GEs in DAG oil did not increase the risk of cancer. However, other dihydroxypropyl agents (besides GEs) are found in edible oils and should also be considered. Alkylating agents (including G) can bind covalently to numerous biomolecules such as proteins or DNA, leading to deleterious effects. The amount and/or content of Hb adducts have been widely applied to dose monitoring in persons exposed to reactive chemicals (Törnqvist and Landin, 1995; Törnqvist *et al.*, 2002; Boogaard, 2002; Ogawa *et al.*, 2006). Exposure to many reactive chemicals besides G, for example styrene, styrene oxide, ethylene oxide, propylene oxide, acrylamide, and acrylonitrile, has been observed to cause Hb adduct formation in humans (Ogawa *et al.*, 2006). Acrylamide and its metabolite, glycidamide, are known animal carcinogens and can

form Hb adducts in humans. The average levels of these *N*-(2-carbamoylethyl)-valine (acrylamide-valine) and *N*-(2-carbamoyl-2-hydroxyethyl)-valine (glycidamide-valine) adducts have been reported to range from 19-76 pmol/g globin and 3-34 pmol/g globin, respectively, in non-smokers (EPA, 2010). Ethylene oxide is also a known animal carcinogen that reacts in a similar manner with biomolecules and forms Hb *N*-2-hydroxyethylvaline (HOEtVal) adducts, with reported levels ranging from 20-50 pmol/g globin in non-smokers (Ogawa *et al.*, 2006). diHOPrVal was present at lower levels than these adducts (2.1-7.5 pmol/g globin, Landin *et al.* 1997, Paper II [Honda *et al.*, 2012]) and although the absolute adduct levels cannot simply be compared, their analysis can help quantify the threats of exposure to reactive chemicals. The available information on Hb adducts suggests that the risk of exposure to other reactive chemicals is greater than the risk of exposure to G for the typical human lifestyle.

3.4.2 Background source of diHOPrVal adducts

Consistent with previous studies of G-Hb adducts in humans (Landin *et al.*, 1996 and 1997), all subjects in the present study (Papers I and II [Honda *et al.*, 2011; 2012]) had some Hb diHOPrVal adducts, regardless of DAG oil ingestion, indicating that they had all been exposed to dihydroxypropylating agents. Characterization of the population distribution profiles of a range of exposure markers, including Hb adducts, would help to identify the potential sources of these agents. The distribution of individual diHOPrVal levels in both the DAG oil-exposed and non-exposed groups was similar to a normal distribution (Fig. 17), whereas occupational exposure to chemicals often exhibits a lognormal distribution (USEPA, 1992, Borjanovic *et al.*, 1999, MHLW, 2003). On the

other hand, the glucose HbA1c adduct shows a normal distribution in healthy subjects (Pradhan *et al.*, 2007, Hanneke, 2011). Since most foods contain glucose and its precursors, people are uniformly exposed to these, resulting in a normal distribution of HbA1c. Although the sample size of our experiment may not be large enough to confirm the distribution profile of diHOPrVal, the results of the present study (Papers I and II [Honda *et al.*, 2011; 2012]) suggested that the donors had been uniformly exposed to dihydroxypropyl agents and that no specific subpopulation had been exposed to high doses of G.

The possible routes to G and diHOPrVal adduct formation from precursors are shown in Fig. 19. Although epichlorohydrin is known to react with Hb to form diHOPrVal, this source is limited to humans with occupational exposure and G is considered the most likely cause of background diHOPrVal. Various food components (anhydro sugars, allyl alcohol, glycerol, glycerol halohydrins, etc.) have been shown to form G, either chemically or by metabolism (Hauschild and Petit, 1956; Hamlet, 1998; Piasecki *et al.*, 1990; Jones, 1975; Patel *et al.*, 1980). Other environmental factors and endogenous metabolites could provide potential sources of G. Ishidao *et al.* (2002) reported that 1-bromopropane can be metabolized to G in the human body, and the involvement of polypropylene oxide as an intermediate was suggested. Although 1-bromopropane and propylene oxide are widely used in chemical products, the level of human G exposure associated with these chemicals is not well characterized, and would need to be explored. G has also been found in tobacco smoke, and elevated diHOPrVal adduct levels in smokers was reported by Landin *et al.* (1997). Since smokers were excluded from the present study (Paper II [Honda *et al.*, 2012]), a dietary source was the most plausible cause of the background diHOPrVal levels observed.

Through analysis of the BDHQ, we identified the food items that were ingested in larger amounts by donors with above median diHOPrVal levels, compared to those with below median diHOPrVal levels. Foods and food components that were related to diHOPrVal adduct levels are shown in Table 5. Among the 70 food items and 97 nutrients examined, western confectionary showed a significant positive correlation with diHOPrVal adduct levels (Pearson's correlation coefficient = 0.291), while meat dishes (simmered food) showed a negative correlation of borderline statistical significance (Pearson's correlation coefficient = -0.217). However, none of the other food items or nutrients exhibited significant relationships with diHOPrVal levels. Moreover, this analysis revealed that the high exposure group (above median diHOPrVal group) ingested 1.66 times more western confectionary than the low exposure group (under median diHOPrVal group) (Welch's t-test, p-value = 0.006). This correlation suggested that G and/or its related compounds may be present in western confectionary, possibly generated by cooking sugars at a high temperature. In contrast, the meat dishes (simmered food) that showed a negative correlation with diHOPrVal adduct levels would probably be cooked at a lower temperature than grilled or fried foods. This result indicated that diHOPrVal adduct levels might be decreased by eating foods cooked at lower temperatures. Thus, in addition to the food items, the method of food preparation should be evaluated to address possible dietary sources of background diHOPrVal levels.

Recently, Eckert *et al.* (2011) reported important findings relating to background levels of G exposure by measuring 2,3-dihydroxypropyl mercapturic acid (DHPMA) in human urine. Hydroxyalkyl mercapturic acids (HAMA) are known to be generated by enzymatic or spontaneous conjugation of endogenous glutathione with electrophilic

substances, and are used as biomarkers for acute exposure to alkylating substances because they are excreted in urine with a relatively short half-life of several hours (Haufroid and Lison, 2005). Eckert *et al.* measured six HAMAs as biomarkers for several alkylating substances (including G) in the urine of 40 smokers and 54 non-smokers in Germany. They found a relatively high background level of DHPMA and a strong correlation with urinary creatinine, regardless of smoking status, indicating an endogenous DHPMA origin from within the body. When we started to investigate G-Hb adduct levels in DAG oil-exposed donors, we were unaware of the utility of urinary DHPMA for acute G exposure assessment, and did not obtain urine samples. Further evaluation of both long-term (G-Hb adduct) and short-term (DHPMA) biomarkers of G exposure would give valuable insights into background G exposure.

3.4.3 Risk assessment

The MOE for GEs found in DAG oil (342) was calculated based on the worst-case scenario, where G was released from GEs perfectly. This was similar to the MOE for acrylamide risk in humans without known chemical exposure (310) and is one twenty-ninth of the MOE of 10,000, considered to "be of low concern from a public health point of view" (EFSA, 2005). In addition, our finding that G-Hb adducts were not increased by daily ingestion of DAG oil indicated that this worst-case scenario was unlikely.

An iMOE of 2,500 would be of low concern and equivalent to a MOE of 10,000, taking species toxicokinetics differences into account (corresponding to 4-fold as the uncertain factor). In short, the iMOE of non-exposed subjects (500) was 20% of the low concern

level. This result indicated a certain level of risk derived from exposure to G-related compounds, regardless of DAG oil ingestion. However, this level was not considered a serious threat to health.

The estimated *iDEL* of G (22 nMh) in humans could be compared to the *in vivo* doses estimated for other reactive chemicals confirmed to be present as background exposure in humans. For example, *iDEL* is estimated to be 2.3 nMh for ethylene oxide (Törnqvist, 1996), and approximately 50 nMh for glycidamide in individuals with high acrylamide intake from food (10-20 times higher intake than the population average) (Vikström *et al.*, 2011). Although G formation in foods has been reported, it may not explain the total estimated oral G exposure. Endogenous G formation and/or exposure to G precursors might be involved, although further investigation is necessary to clarify this. Until the sources of G and their contributions to the *in vivo* dose have been identified, we cannot exclude other sources of the diHOPrVal adduct, even though dietary G seems to be the most plausible source. In this way, measurement of Hb adducts enables us to estimate the *in vivo* dose of chemicals to which one is exposed at very low levels. This method has considerable advantageous over more conventional analyses of chemicals/metabolites in tissue samples.

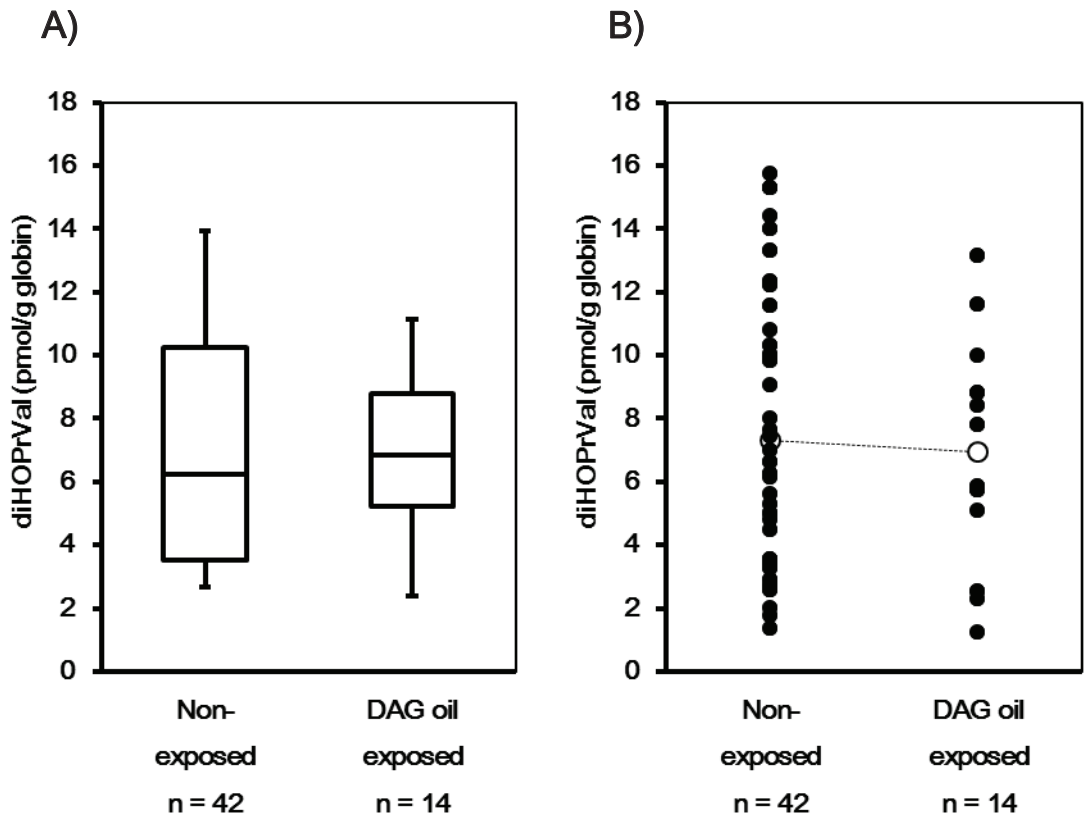

Fig. 17 Distribution of diHOPrVal levels in the matched case-control study

A) Closed circles represent individual Hb diHOPrVal adduct levels. Open circles represent the average diHOPrVal level in each group. B) diHOPrVal distribution in each study group is shown as a box plot showing the median adduct level (horizontal line), with the 10th, 25th, 75th, and 90th percentiles presented as vertical boxes and error bars. All human blood samples were obtained from non-smokers.

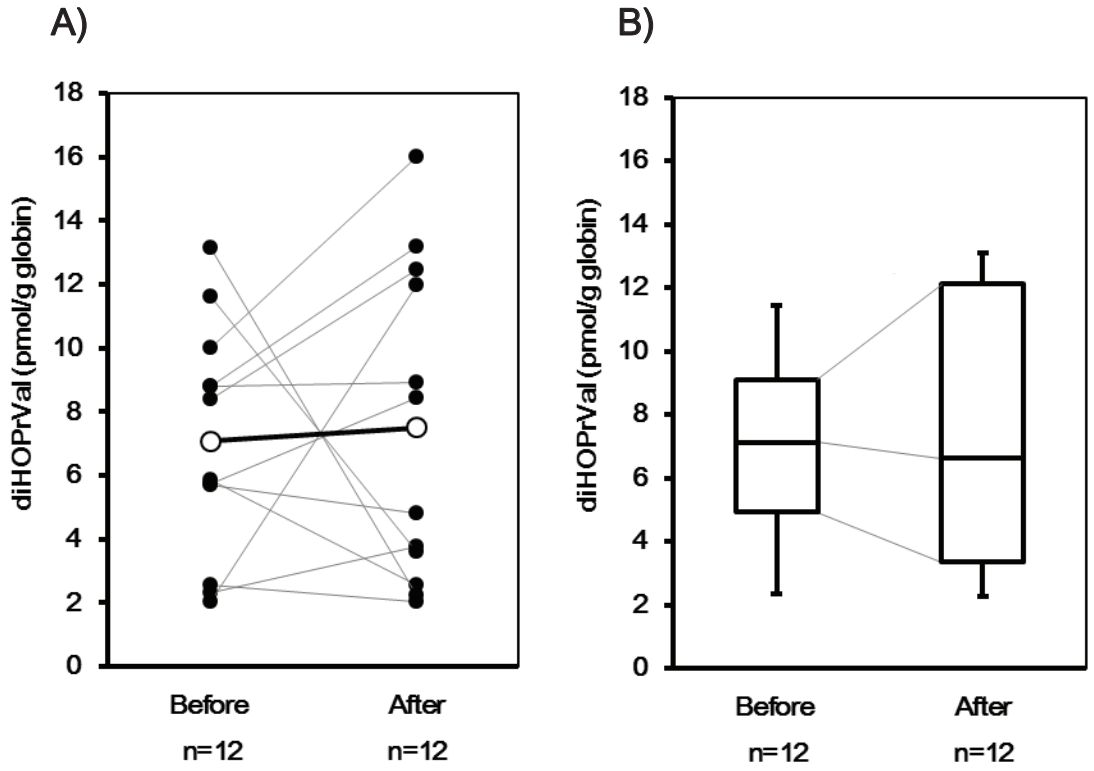

Fig. 18 Distribution of diHOPrVal levels before and after discontinuing the use of DAG oil

A) Closed circles represent individual Hb diHOPrVal adduct levels. Open circles represent the average levels of diHOPrVal in each group. B) diHOPrVal distribution is illustrated as a box plot for each group, showing the median adduct level (horizontal line), with the 10th, 25th, 75th, and 90th percentiles presented as vertical boxes and error bars. All human blood samples were obtained from non-smokers.

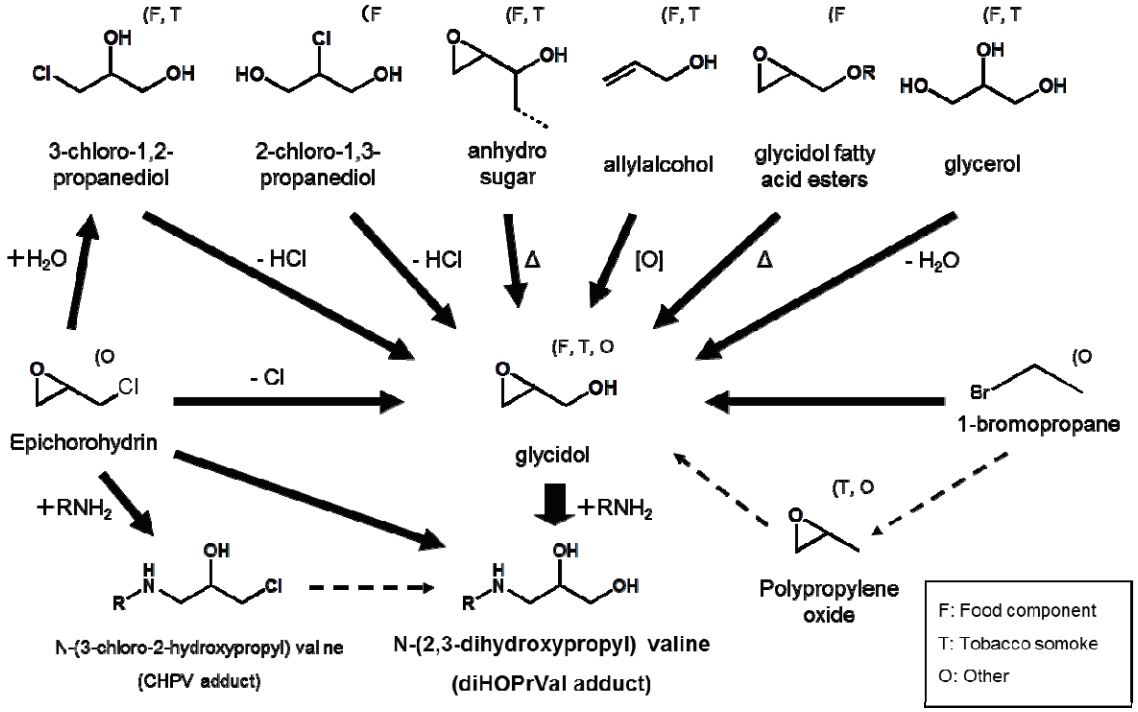

Fig. 19 Possible routes to diHOPrVal formation

Table 2 Individual diHOPrVal levels and relevant information on the matched case-control study subjects

Block	Case (DAG oil-exposed donor)					Control (Non-exposed donor)				
	Donor code	Sex	Age (year)	diHOPrVal (pmol/g globin)	Assumed edible oil ingestion (g/day)	Donor code	Sex	Age (year)	diHOPrVal (pmol/g globin)	Assumed edible oil ingestion (g/day)
						C7	F	44	2.0	9.9
I	E1	F	42	1.2	5.5	C8	F	43	5.3	6.7
						C9	F	44	13	11.5
						C10	M	46	7.0	15.8
II	E2	M	46	5.1	5.9	C11	M	47	2.7	14.3
						C12	M	45	10	10.6
						C13	M	32	3.5	23.7
III	E3	M	34	2.5	10.1	C14	M	34	9.8	12.2
						C15	M	32	11	18.3
						C16	M	27	3.5	13.8
IV	E4	M	28	2.3	15.3	C17	M	28	6.2	7.6
						C18	M	29	7.4	13.0
						C19	M	50	10	7.1
V	E5	M	53	5.7	0.8	C20	M	52	15	5.2
						C21	M	52	6.2	4.7
						C22	M	44	14	21.5
VI	E6	M	43	5.9	18.3	C23	M	43	9.1	11.6
						C24	M	41	8.0	14.8
						C25	M	27	12	27.2
VII	E8	M	25	8.8	15.6	C26	M	25	2.7	11.0
						C27	M	25	3.5	26.7
						C28	M	35	7.6	12.8
VIII	E9	M	37	13	19.9	C29	M	38	1.4	11.0
						C30	M	37	2.6	18.9
						C31	M	28	4.5	4.6
IX	E10	M	28	10	5.6	C32	M	28	3.4	9.3
						C33	M	29	3.3	7.8
						C34	F	29	5.6	6.4
X	E11	F	28	8.8	10.9	C35	F	28	5.0	5.5
						C36	F	26	1.7	20.7
						C37	F	38	15	3.0
XI	E12	F	36	12	9.1	C38	F	38	12	6.9
						C39	F	39	14	15.2
						C40	M	41	6.2	11.7
XII	E13	M	41	8.4	9.4	C41	M	44	3.5	10.7
						C42	M	43	10	11.6
						C43	F	47	6.6	12.0
XIII	E14	F	45	5.7	5.6	C44	F	48	4.8	10.2
						C45	F	47	6.1	10.4
						C46	F	30	12	12.5
XIV	E15	F	30	7.8	9.6	C47	F	30	16	13.4
						C48	F	30	2.9	11.5

Table 3 Results of two-way ANOVA of the matched case-control study

Group	Least square mean	95% confidence interval		p-value	
		lower	upper	DAG oil ingestion	Block (Age and Sex)
Case (DAG oil-exposed donor)	6.9	4.9	9.0	0.744	0.152
Control (Non-exposed donor)	7.3	6.1	8.5		
Mean difference	-0.39	-2.8	2.0		

Table 4 Individual diHOPrVal levels before and after discontinuation of DAG oil

Donor code	Sex	Age (year)		diHOPrVal (pmol/g globin)		Assumed edible oil ingestion (g/day)	
		2009-2010	2011	2009-2010	2011	2009-2010	2011
Control (Non-exposed donor)							
C1	M	51	53	13	1.3	8.0	9.5
C2	M	43	45	5.6	14	9.2	8.4
C3	F	46	48	8.7	16	1.4	6.4
C4	M	45	47	6.0	2.3	5.0	10.5
C5	M	27	28	5.2	15	8.6	7.7
C6*	M	48	-	4.4	-	6.0	-
Case (DAG oil exposed donor)							
E1*	F	42	-	1.2	-	5.5	-
E2*	M	46	-	5.1	-	5.9	-
E3	M	34	36	2.5	2.0	10.1	10.0
E4	M	28	29	2.3	3.8	15.3	17.9
E5	M	53	55	5.7	4.8	0.8	2.2
E6	M	43	45	5.9	2.5	18.3	24.2
E7†	M	47	49	2.0	12	9.6	14.0
E8	M	25	26	8.8	13	15.6	19.5
E9	M	37	38	13	2.2	19.9	15.3
E10	M	28	29	10	16	5.6	8.9
E11	F	28	29	8.8	8.9	10.9	16.6
E12	F	36	38	12	3.6	9.1	6.5
E13	M	41	42	8.4	12	9.4	9.1
E14	F	45	46	5.7	8.4	5.6	12.3
E15*	F	30	-	7.8	-	9.6	-

2009-2010: before discontinuing the use of DAG oil

2011: after discontinuing the use of DAG oil

* excluded from analysis

† smoker

Table 5 Foods that correlated with Hb diHOPrVal adduct levels

Foods and components	Daily ingestion ratio	Welch's t-test p-value	Pearson's correlation coefficient
Foods			
Positive correlation			
western confectionary	1.66	0.006	0.291
Negative correlation			
meat dish (simmered food)	0.75	0.064	-0.217

Summary and overall conclusion

Firstly, we established an analytical method for measurement of G-Hb diHOPrVal adducts using GC-NCI-MS/MS. This method showed good between-run and within-run reproducibility and the tentative LOQ was equivalent to previously-published information (Landin *et al.*, 1996). In addition, diHOPrVal adducts were identified in all non-exposed rat samples, indicating that this method was sensitive and therefore applicable to the analysis of extremely low exposure to G related compounds in daily life.

Secondly, to investigate the significance of diHOPrVal as an *in vivo* marker of G exposure, the kinetics of diHOPrVal formation were investigated in rats that had been orally administered with G. diHOPrVal Hb adducts showed G dose-dependent formation, chemical stability, and sensitivity. Furthermore, we estimated the *in vivo* dose of G based on diHOPrVal levels and compared this to previous AUC data. These results showed that diHOPrVal provided a powerful tool to measure *in vivo* G exposure and assess the associated risk.

Thirdly, we used diHOPrVal quantification to assess G exposure in study subjects who had ingested DAG oil containing small amounts of GEs. This matched case-control study found comparable diHOPrVal levels in the DAG oil-exposed and non-exposed groups. Furthermore, discontinuation of DAG oil usage did not affect diHOPrVal levels. These results suggested minimal G exposure in DAG oil-exposed subjects, although the size of the population evaluated was limited. To assess the risk posed by chemical

exposure fully, other sources of reactive chemicals encountered in daily life need to be explored.

Finally, we have suggested a new, more realistic, approach to cancer risk assessment using the *iDEL* derived from the Hb adduct levels. It is expected that this method will contribute to the development of more accurate risk assessments based on realistic estimates of internal exposure.

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