Journal of Chromatography A, 1218 (2011) 4389–4394

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Quantitative analysis by liquid chromatography–tandem mass spectrometry of glycidamide using the cob(I)alamin trapping method: Validation and application to *in vitro* metabolism of acrylamide

Hitesh V. Motwani, Margareta Törnqvist*

Department of Materials and Environmental Chemistry, Stockholm University, SE-106 91, Stockholm, Sweden

ARTICLE INFO

Article history:
Received 17 December 2010
Received in revised form 22 February 2011
Accepted 9 May 2011
Available online 13 May 2011

Keywords:
Glycidamide
Cob(I)alamin
Alkylcobalamin
LC–MS/MS
Liver S9

ABSTRACT

Glycidamide (GA) is the epoxy metabolite of acrylamide (AA). A sensitive analytical method for quantitative measurement of GA from *in vitro* metabolism studies is useful in several contexts, e.g. in studies of enzyme kinetics in different species and factors influencing the metabolism of AA to GA. It is however difficult to analyse compounds like GA, mainly due to their inherent reactivity. In the present study cob(I)alamin {Cbl(I)}, a reduced form of vitamin B_{12}, was used for trapping of GA. Cbl(I) can react with electrophilic species, such as an epoxide, 10^5 times faster than standard nucleophiles. The trapping of GA by Cbl(I) results in the formation of an alkylcobalamin (GA-Cbl) that was used for quantitative analysis of the epoxide. The alkylcobalamin was analysed by LC–MS/MS using an electrospray ionization source in the positive ion mode. The Cbl(I) method was validated for measurement of GA in liver S9 fractions from human and rat. GA levels down to 0.01 μM were measured in the S9 fractions, providing a sensitivity that was ca. 100 times higher than that earlier estimated by the Cbl(I) method for measurement of other (e.g. butadiene) epoxides. Compared to current analytical methods for measurement of GA, the Cbl(I) method was 10–100 times more sensitive. The method was applied to quantify GA formed from the metabolism of AA in liver S9 from human and rat.

1. Introduction

Acrylamide (AA) is a neuro- and reproductive toxicant, and a multisite carcinogen in rodents following chronic high-dose exposures [1,2]. IARC has classified AA as a probable human carcinogen (group 2A) [3]. Our earlier discovery of AA in fried and baked foods [4,5] raised concern about the potential health effects associated with the widespread exposure to AA from cooked foods [1,2,6]. AA is also an important industrial chemical with an annual worldwide production estimated to >200 million kg. It is used in the manufacturing of polyacrylamide and grouting agents.

AA has been shown to metabolize to the reactive epoxide glycidamide (GA) [7,8], by cytochrome P450 (P450) 2E1 [9] (Fig. 1). Both AA and GA are detoxified by glutathione conjugation and GA is also detoxified by hydrolysis, probably catalysed by epoxide hydrolase [10,11]. Both AA and GA are electrophilic compounds, though GA has a considerably higher reactivity than AA towards nucleophilic sites in DNA and is shown to form DNA adducts *in vivo* [12,13]. Accordingly, GA is mutagenic (e.g. [14]) and assumed to be the ultimate cancer initiating agent from AA exposure [15,16]. The metabolism of AA to GA varies between different species [17–20] and is influenced by factors such as dietary compounds, e.g. garlic [21]. For the extrapolation of risk to humans from response in laboratory animal experiments, e.g. in tests of carcinogenicity, it is essential to know the species differences in metabolism.

The metabolic formation of GA from AA with respect to interspecies difference have been studied *in vivo*, using MS techniques to measure stable adducts to haemoglobin [16,20,22] or DNA [12,13]. Metabolism can also be studied *in vitro* using, e.g. microsomes or S9 liver fractions. A pre-requisite for the detection and quantification of GA formed in an *in vitro* metabolic system is a sensitive and accurate analytical method. Relatively high sensitivity and recovery in the measurement of GA was obtained by Twaddle et al. [17]. Using a SPE-LC/MS/MS method they obtained a LOQ of 0.1 μM and 85% recovery of GA in analysis of serum samples from AA-exposed rodents [17]. Besides LC–MS/MS [17,21,23], GA has also been analysed by NMR [24] and HPLC-UV (at 200 nm) [25] in biological matrix, whereby the compound is usually analysed either directly by head-space technique or after solid or liquid phase extraction. However, due to the hydrophilicity, low molecular weight and reactivity of GA, these procedures are not ideal for the purpose of accurate quantification.

* Corresponding author at: Department of Materials and Environmental Chemistry, Environmental Chemistry Unit, Stockholm University, SE-106 91 Stockholm, Sweden. Tel.: +46 8 163769; fax: +46 8 152187.
E-mail address: margareta.tornqvist@mmk.su.se (M. Törnqvist).

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doi:10.1016/j.chroma.2011.05.008

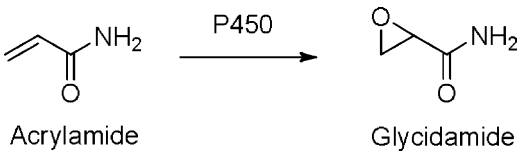

Fig. 1. Metabolism of acrylamide to glycidamide.

An electrophilic compound like GA could be stabilized by derivatization with a nucleophile, to improve the accuracy, chromatography, sensitivity and specificity of the analysis. Amongst the derivatizing agents applied to date are 3,4-dichlorobenzenethiol [26], *p*-nitrobenzylpyridine [27], angiotensin II [28], glutathione [29] and 2-mercaptobenzoic acid [30]. However, due to their low nucleophilic power, they require long reaction time and hence are not suitable for metabolic studies. For instance, derivatization of GA with 2-mercaptobenzoic acid takes about 12 h [30]. Previously, we have introduced cob(I)alamin (Cbl(I)) as a reagent for rapid (ca. 10^5 times faster than standard nucelophiles [31]) trapping of butadiene epoxides formed during *in vitro* metabolism studies that result in formation of specific alkylcobalamins from each epoxide [32,33]. The alkylcobalamins are stable, have high molecular masses and are polar complexes, which makes them suitable for analysis by LC-UV and LC–MS [34,35]. In the present study, the Cbl(I) trapping method was adapted and developed with respect to the LC–MS/MS conditions for analysis of GA formed in an *in vitro* metabolic system, and thereby enhancing the sensitivity of the method. The Cbl(I) method for analysis of GA was validated and applied to measure the metabolic formation of GA from AA in human and rat S9 liver fractions.

2. Experimental

2.1. Chemicals and other materials

Hydroxocobalamin hydrochloride (OH-Cbl·HCl), cobalt(II) nitrate, and β-nicotinamide adenine dinucleotide phosphate sodium salt (NADP) were obtained from Sigma (St. Louis, MO, USA). Glucose-6-phosphate, magnesium sulfate, potassium chloride, sodium borohydride, trifluoroacetic acid and *rac*-propylene oxide (PO) were obtained from Sigma–Aldrich Sweden AB (Stockholm, Sweden). *rac*-Glycidamide (GA) was obtained from Toronto Research Chemicals Inc., Canada. Acrylamide (AA) was obtained from Merck, Germany. GA and PO were kept ice cold until use to prevent hydrolysis under ambient conditions. All solvents used were of HPLC grade. Human and rat liver S9 fraction (14.4 and 32.4 mg proteins/mL, respectively) were purchased from Moltox (Boone, NC, USA). Tests for P450 activity, presence of adventitious agents and promutagen activation were provided by the supplier (Moltox).

Caution: GA and PO are potentially carcinogenic compounds. They were handled in a ventilated hood and destroyed immediately after use by 1 M aqueous H_2SO_4.

Note: Cobalamins were protected from light by performing reactions in amber coloured vials or by using aluminium foil shields when needed.

2.2. Liquid chromatography

An automated switch-valve was used between a LC 20 system (Shimadzu Prominence, Kyoto, Japan) and an API 3200 Q trap triple quadrupole mass spectrometer (MS) that was equipped with a TurboIonSpray® interface, AB SCIEX (Concord, ON, Canada). An ACE 5 C18 10 mm × 1 mm precolumn from the LC was connected to the switch, which was coupled to the MS via an ACE 5 C18 150 mm × 1 mm analytical column. A gradient using two mobile phase systems of 5% acetonitrile (A) and 70% acetonitrile (B), respectively, in water with 0.1% trifluoroacetic acid in each was used for elution of the alkylcobalamins. A gradient was applied from 5% B to 20% B in 10 min, then to 60% B in 7 min, followed by an increase to 100% B in 1 min which was held for 5 min before re-equilibrating the column with the initial mobile phase. The injection volume was 10 μL and the flow rate was 150 μL/min. Immediately following injection, the automatic switch allowed the excess OH-Cbl and other waste, such as polar compounds in the S9 from the injected sample, to pass the precolumn and enter the waste. After 5 min, the valve was switched to the eluting position, thus diverting the eluent to the analytical column, where the alkylcobalamins were separated, followed by MS detection.

2.3. Mass spectrometry

The electrospray ionization (ESI) source of the MS was operated in the positive ion mode. Acquisition and data processing were performed using the Analyst software version 1.5 from AB SCIEX. Instrument settings for the MS with the offset values in V: declustering potential 25 V, entrance potential 7.5 V, collision energy 30 V, nebulizer gas (N_2) 15 arbitrary units (au), curtain gas (N_2) 40 au, collision gas (N_2) 5 au, ion spray voltage 5500 V and vaporizing temperature 400 °C. The MS was first operated in full scan mode, and then product ion scan (PIS) mode was performed to determine the fragments of the alkylcobalamin from GA (GA-Cbl, *m/z* 709.8). Quantitative analysis of processed samples was performed using multiple reaction monitoring (MRM) mode with the following transitions: GA-Cbl *m/z* 709.8 → 665.5, PO-Cbl (alkylcobalamin from PO (internal standard)) *m/z* 695.2 → 665.5.

2.4. Preparation of Cbl(I)

Cbl(I) was prepared according to the method described by Haglund et al. [32]. Briefly, in an amber vial sealed with septum, aq. OH-Cbl (5 mM, 200 μL) and cobalt(II) nitrate (25 mM, 20 μL) were mixed under argon for 10 min. Aqueous sodium borohydride (150 mM, 50 μL) was added which resulted in the generation of Cbl(I).

2.5. Formation of GA-Cbl

An aqueous solution of GA (2 μM, 200 μL) and PO (3 μM, 200 μL) was made. Three hundred microliters from this solution was added to previously prepared Cbl(I) under an inert atmosphere, which resulted in the formation of GA-Cbl and PO-Cbl. After 20 min air was passed into the reaction mixture, to oxidize any remaining reduced cobalamin. The solution was then analysed by LC–MS/MS.

2.6. Preparation of S9 mixture

To human or rat S9 fraction (150 μL) at 4 °C was added aq. KCl (0.15 M, 300 μL), $MgSO_4$ (80 mM, 150 μL), glucose-6-phophate (50 mM, 150 μL), and NADP (40 mM, 150 μL), and the volume was made up to 1.5 mL with pH 7.5 phosphate buffer. P450 activity in the S9 was assessed by measuring the rate of conversion (oxidation) of 1,2-epoxy-3-butene to 1,2:3,4-diepoxybutane as described in our earlier work [33] (data not shown).

2.7. Preparation of calibration standard curve

To S9 mixtures (1.5 mL each) was added GA (20 μL) to give the following concentrations: 0.01, 0.04, 0.4, 2, 10, 20, 50, 100, 200 μM. Two hundred microlitres of the S9 mixture was added to PO (3 μM, 200 μL) in ice cold methanol, mixed, and centrifuged at 500 × *g* for

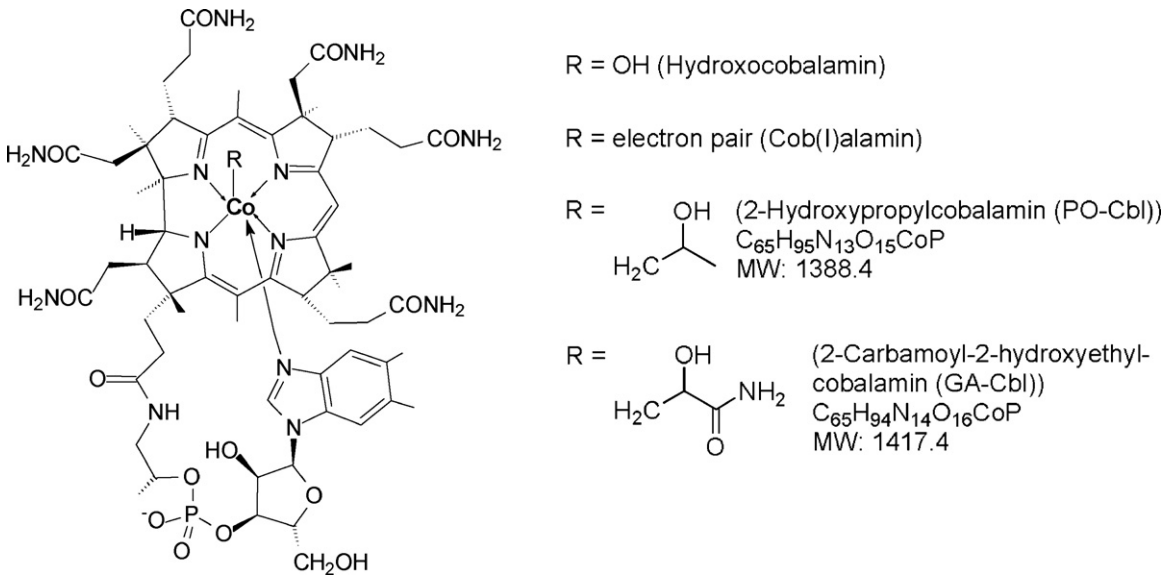

Fig. 2. Cobalamin structures with molecular formula and molecular weight (MW) of the alkylcobalamins (PO-Cbl and GA-Cbl).

4 min to precipitate the proteins. The supernatant (300 μL) was added to previously prepared Cbl(I) solution to form respective alkylcobalamins, GA-Cbl and PO-Cbl, which were quantified by LC–MS/MS using the MRM mode.

2.8. Cbl(I) for determination of GA

2.8.1. Linearity test

The test of linearity was performed by preparing the calibration curve for different concentrations of GA over a range of 0.01–200 μM ($n = 9 \times 4$). From the MRM analysis the areas under the peaks corresponding to GA-Cbl and PO-Cbl were measured, and their ratio (Area GA-Cbl/Area PO-Cbl) were plotted against the respective GA concentrations.

2.8.2. Sensitivity test

The limit of quantification (LOQ) of GA was experimentally determined as the lowest concentration of the linearity test with accuracy (expressed as relative error (RE)) ± 20% and precision (expressed as relative standard deviation (RSD)) ≤ 20%. The LOQ was acceptable with a signal to noise (S/N) ratio of 10 and for the limit of detection (LOD), a S/N of 3 was considered acceptable.

2.8.3. Preparation of quality control S9 samples

Four S9 quality control (QC) samples were prepared in the same way as mentioned for the calibration standard curve over a range of GA concentrations (0.04, 2, 50 and 200 μM). The concentrations were chosen to evaluate the values at low, moderate and high concentrations of the calibration curve. These were used in the assessment of the specificity, analytical recovery, stability, intra- and inter-day accuracy and precision.

2.8.4. Specificity

Four blank S9 mixture (without added GA) and the four QC S9 samples with GA (0.04–200 μM) were analysed and compared for the presence of GA-Cbl. This would indicate if the presence of other substances in the S9 fraction interferes with the measurement of GA as GA-Cbl.

2.8.5. Analytical recovery and stability

The MRM response of GA-Cbl obtained from QC S9 samples with different concentrations of GA (0.04–200 μM, $n = 4 \times 4$) was compared with the response of equivalent GA concentration in S9 matrix free samples. Average values were taken for each concentration and the analytical recovery was calculated as percentage.

Stability of GA-Cbl and PO-Cbl at ca. −20 °C (storage condition) was assessed by analyzing four QC samples for each concentration (0.04–200 μM) when freshly prepared and at the end of 30 days of storage. The percentage of difference between the mean concentrations of GA was calculated.

2.8.6. Intra-day and inter-day accuracy and precision

Accuracy and precision were expressed as percent RE and percent RSD, with ±15% and ≤15% acceptance limits, respectively. Intra-day and inter-day accuracy and precision of the method were determined by analyzing the QC S9 samples having GA concentration over a range 0.04–200 μM ($n = 4 \times 6$) in a day and in 3 different days over a month, respectively. The significance of the acceptance level, concentration range and number of determinants has been discussed earlier [36–38].

2.9. In vitro metabolic formation of GA

Human or rat S9 mixture was prepared as described above. The mixture in duplicates was incubated for 5 min at 37 °C prior to addition of AA (1 mM). An aliquot (200 μL) of the incubation mixture was taken out at different time intervals (0–90 min) and was added to PO (3 μM, 200 μL) in ice cold methanol, mixed, and centrifuged at 500 × *g* for 4 min. Three hundred microliters of supernatant was added to previously prepared Cbl(I) and the reaction was continued for 20 min under inert atmosphere. Air was passed into the mixture for 5 min to quench the reaction and the samples were analysed by LC–MS/MS. The concentration of GA was extrapolated from the calibration curve. In control studies incubations were performed by the same method but in the absence of NADP, and in the absence of AA.

3. Results and discussion

3.1. Formation of an alkylcobalamin from GA

It is known that Cbl(I) instantaneously reacts with electrophilic substances, such as an epoxide, by an S_N2-type mechanism that results in the formation of an alkylcobalamin [31,32,39] (for cobal-

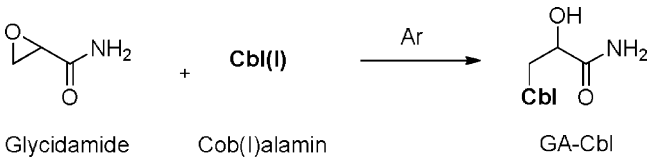

Fig. 3. Formation of GA-Cbl.

amin structures see Fig. 2). In the present study Cbl(I) was prepared by reduction of OH-Cbl (i.e. cob(III)alamin) using sodium borohydride and a reduction catalyst, cobalt(II) nitrate. The reactions were carried out under an inert atmosphere to prevent back-oxidation of Cbl(I) to cob(II)alamin or cob(III)alamin. GA-Cbl was formed by addition of GA to a Cbl(I) solution under a stream of argon (Fig. 3). PO was used as an internal standard, which also reacts with Cbl(I) by the same mechanism to give PO-Cbl. Considering the nucleophilic strength of Cbl(I) (ca. 10 on the Swain-Scott scale [39,40]), there is a potential of reducing the reaction time (20 min), which however was not explored in the present study.

As shown in Fig. 3, Cbl(I) is proposed to attack by nucleophilic displacement (S_N2-type) at the less hindered carbon (C1) in GA to give GA-Cbl. The bulky Cbl(I) is expected to be less reactive towards the more substituted carbon (C2) of the epoxide [39,41]. Further, a nucleophilic attack on C2 would result in a secondary alkylcobalamin that are particularly unstable as they could decompose spontaneously yielding the corresponding olefin and a reduced form of cobalamin [39,42]. Thus the formation of regioisomers as products in the reaction shown in Fig. 3 is highly unlikely. Moreover, earlier studies on reactions of GA with N-terminal valine in haemoglobin or guanine in DNA have also shown only one reaction product by the nucleophilic attack on C1 of GA [12,22].

Once earlier, the formation of GA-Cbl was reported in a reaction-kinetic study, wherein the alkylcobalamin was analysed by LC-UV [41]. It was shown that GA was hydrolysed with a half-life of 43 h in water (pH 6) at 37 °C [41]. Relatively high concentrations of GA (1–5 mM) were used and sensitivity in the analysis of GA-Cbl was thus not an issue in the earlier study [41]. Whereas, in the present work a sensitive LC–MS/MS method was developed for the analysis of GA-Cbl in biological matrix (Sections 3.2 and 3.3) aiming at applications in quantitative metabolism studies (Section 3.4).

3.2. Characterization of GA-Cbl by LC–MS/MS

The MS spectra of GA-Cbl in the full scan positive ion mode showed the molecular ion at *m/z* 1418 $[GA\text{-}Cbl+H]^+$ and a doubly protonated base peak at *m/z* 709.8 $[GA\text{-}Cbl+2H]^{2+}$. PIS performed on the base peak *m/z* 709.8 gave the cobalamin fragments *m/z* 665.5 (100%), 972.5 (32%) and 359 (20%). The major fragment *m/z* 665.5 $[Cbl+2H]^{2+}$, was obtained by loss of the alkyl (GA) moiety from the cobalt of cobalamin. For structures of cobalamin fragments *m/z* 359 and 972.5, see Ref. [43].

MRM mode was used for quantification, whereby the precursor ion to product ion transition used for GA-Cbl and PO-Cbl were *m/z* 709.8 $[GA\text{-}Cbl+2H]^{2+}$ to 665.5 $[Cbl+2H]^{2+}$ and *m/z* 695.2 $[PO\text{-}Cbl+2H]^{2+}$ to 665.5 $[Cbl+2H]^{2+}$, respectively. This is exemplified in Fig. 4, which was obtained from GA (2 μM) and PO (3 μM) (cf. Section 2.5). The double peaks of GA-Cbl and PO-Cbl in Fig. 4 are attributed to a pair of diastereoisomers of the alkylcobalamins formed from the respective racemic epoxides, GA and PO (cf. [34,35]). The possibility of further separation of the double peaks was not tested in the present work.

3.3. Cbl(I) for determination of GA from S9

From quantitative MRM analysis of the analytes, GA-Cbl and PO-Cbl, the concentration of GA in a sample matrix was extrapolated using a calibration curve. There are several variables such as reduction of OH-Cbl, reaction time, matrix effect, chromatographic separation, MS detection and quantification that could influence the analysis. Considering these variables, the validation of the Cbl(I) method for determination of GA concentration, from the sample matrix of S9, included test for linearity, sensitivity, specificity, recovery, stability, accuracy and precision. These are based on the characteristics that should be taken into account during the validation of an analytical method [36–38].

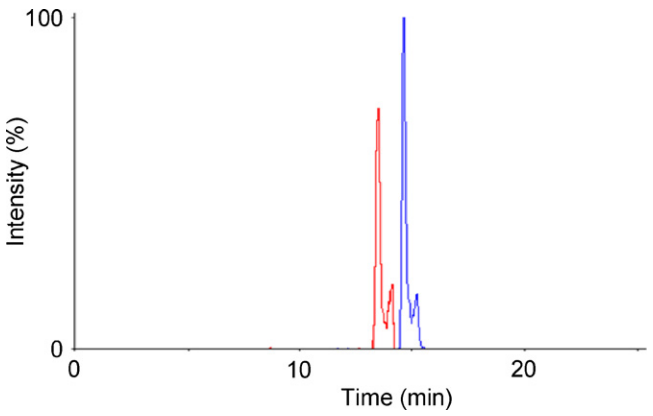

Fig. 4. LC-ESI^+-MRM chromatogram showing GA-Cbl (left peak) and PO-Cbl (right peak). The double peaks represent the respective diastereomers.

3.3.1. Linearity

Test for linearity was performed to evaluate the efficiency of the reaction between Cbl(I) and GA. Different concentrations of GA (0.01–200 μM) were added to S9 fractions under ice cold conditions. Aliquots were removed, mixed with internal standard (PO), centrifuged and the supernatant was added to Cbl(I) solutions. The analytes were quantified by LC–MS/MS in the MRM mode. A linear and reproducible relationship was observed between the peak area ratio of GA-Cbl to PO-Cbl and GA concentration for a 9-point calibration curve (Fig. 5).

3.3.2. Sensitivity

Using the Cbl(I) method with LC–MS/MS analysis, the LOQ of GA from the S9 mixture was determined to be 0.01 μM, with S/N of 10 and injection volume of 10 μL. The LOQ concentration had an acceptable accuracy (10.8% RE) and precision (16.7% RSD) for the study (Table 1). The LOD was estimated to be 0.001 μM.

The LOQ of GA obtained herein using Cbl(I) is 10–100 times lower than that of previous methods for analysis of GA [17,23–25]. Furthermore, the sensitivity for analysis of GA is ca. 100 times higher than for the analysis of 1,2:3,4-diepoxybutane in our earlier

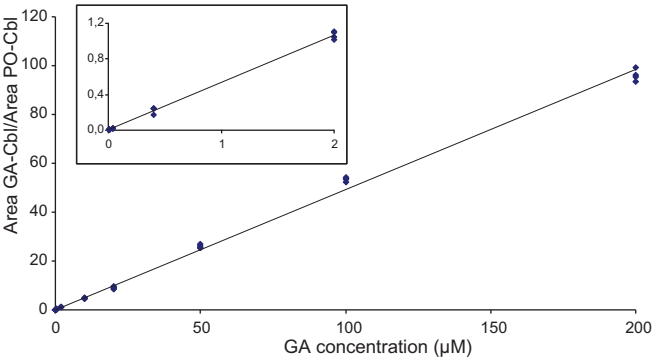

Fig. 5. Calibration curve ($n = 9 \times 4$; $r^2 = 0.996$) for GA, analysed as GA-Cbl. Insert shows the lower GA concentration (0.01–2 μM) region.

Table 1
Nominal GA concentration and back calculated calibration curve concentrations of GA from S9 matrix ($n = 9 \times 4$).

Nominal GA concentration (μM)	Estimated GA concentration (μM)	SD	RE%	RSD%
0.01	0.0111	0.0019	10.8	16.7
0.04	0.0454	0.0049	13.6	10.8
0.4	0.461	0.069	15.3	15.0
2	2.17	0.086	8.65	3.97
10	9.67	0.25	−3.33	2.61
20	18.4	0.89	−8.23	4.88
50	52.5	1.6	5.03	3.03
100	109	1.8	8.57	1.68
200	195	5.0	−2.37	2.57

work [32] using the Cbl(I) trapping method. The higher sensitivity in the present study compared to our earlier work on butadiene epoxides was because of further optimization of the LC–MS/MS parameters (cf. Sections 2.2 and 2.3).

3.3.3. Specificity

Blank samples of S9, i.e. without added GA, were prepared (cf. Section 2) in the same way as QC samples (0.04–200 μM, $n = 4$). No detectable peaks were observed in any of the blank samples at the retention time of GA-Cbl (13 min) as observed in the QC samples. This along with the use of MRM transitions in the LC–MS/MS analysis were considered as acceptable specificity and indicated that substances in the S9 mixture do not interfere with the measurement of GA-Cbl.

3.3.4. Analytical recovery and stability

The recovery of GA from the QC samples (0.04–200 μM, $n = 4 \times 4$), relative to the matrix free samples with same concentration, was calculated to be in the range 94–110% and the average recovery was 102%. Similarly, the average recovery of PO (3 μM, $n = 4$) was determined to be 92%.

In the stability test, the percent deviation of the mean concentration of GA after analysis of the QC samples ($n = 4 \times 4$) following storage at ca. −20 °C for 30 days was ±3% from that of freshly prepared QC samples. This suggested that the alkylcobalamins can be stored at −20 °C for at least up to a month.

3.3.5. Intra-day and inter-day accuracy and precision

Accuracy and precision of analyses performed within a day (intra-day) or within a period of days (inter-day) were determined. Intra-day accuracy and precision of the method ranged from −2.1 to 9.2% and from 2.1 to 5.1%, respectively (Table 2). Inter-day accuracy and precision of the method ranged from −6.0 to 10.2% and from 1.6 to 12.1%, respectively (Table 3).

3.4. In vitro metabolic formation of GA

The Cbl(I) method was applied in the present study for determination of GA concentration from *in vitro* metabolism of AA. The substrate, AA (1 mM), was incubated in human or rat S9 liver fractions. Samples were collected over time intervals (0–90 min), mixed with internal standard (PO) and centrifuged. The supernatant was added to Cbl(I) solution under anaerobic condition and then analysed by LC–MS/MS. The MRM chromatogram was similar to that shown in Fig. 4, most likely because AA metabolizes to give two enantiomers of GA (cf. [2]) and thus two diastereomers of the alkylcobalmin. Area under the peaks corresponding to GA-Cbl and PO-Cbl were measured and concentration of GA at different time points was extrapolated from calibration curve. In both human and rat S9, it was observed that the amount of GA formed under the incubation condition increased linearly up to 20 min (Fig. 6). By 80 min, the amount of GA formed was higher in the rat (10 μM) than in human (7.8 μM). In control studies without NADP or without AA, GA-Cbl was not detected. This approach could be applied in metabolism studies, e.g. in the determination of enzyme kinetic parameters, V_{max} and K_m, associated with the metabolism of AA to GA (cf. [33]).

Table 2
Intra-day accuracy and precision ($n = 4 \times 6$).

Nominal GA concentration (μM)	Estimated GA concentration (μM)	SD	RE%	RSD%
0.04	0.0422	0.0021	5.44	5.07
2	2.18	0.082	9.16	3.76
50	52.6	1.4	5.22	2.65
200	196	4.1	−2.06	2.07

Table 3
Inter-day accuracy and precision (for each day $n = 4 \times 6$).

Nominal GA concentration (μM)	Estimated GA concentration (μM)	SD	RE%	RSD%
Day 1				
0.04	0.0440	0.0045	9.92	10.3
2	2.19	0.083	9.67	3.80
50	54.5	3.3	8.90	6.03
200	189	3.0	−5.28	1.59
Day 2				
0.04	0.0437	0.0053	9.33	12.1
2	2.20	0.14	9.84	6.23
50	53.4	2.3	6.89	4.28
200	188	8.8	−6.04	4.69
Day 3				
0.04	0.0425	0.0039	6.20	9.08
2	2.20	0.10	10.2	4.47
50	54.1	2.3	8.11	4.24
200	189	3.8	−5.32	2.01

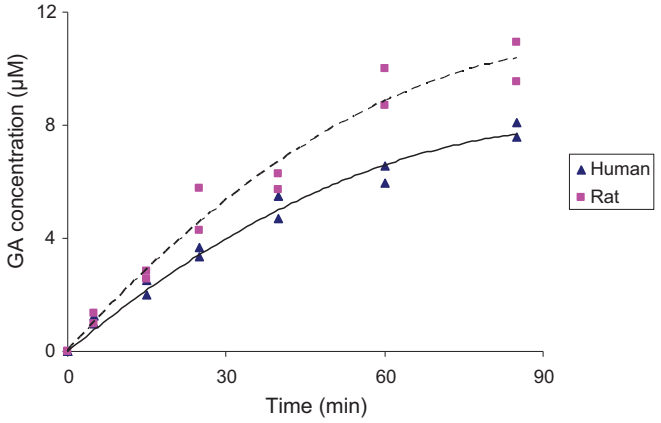

Fig. 6. Formation of GA following incubation of AA (1 mM) in human (▲) and rat (■) liver S9.

4. Conclusions

Species differences in metabolism of AA have been studied *in vivo* by analysis of stable reaction products, adducts, formed with haemoglobin in blood [16,20,22] or DNA [12,13]. It is, however, desirable to reduce animal experiments and to have access to methods for quantitative studies of metabolism *in vitro*. In this context electrophilically reactive metabolites such as GA, which are potential genotoxic agents, constitute an analytical challenge. In the present study, for measurement of concentration of GA in S9 liver

fraction, an analytical method was developed that was based on analysis of alkylcobalamins by LC–MS/MS. For separation of the alkylcobalamins from the waste, an on-line clean-up procedure was used that consisted of a pre-column and a column switch technique. The analytical method was validated in detail by assessing linearity, sensitivity, specificity, recovery, stability, accuracy and precision. The method was applied for the determination of GA concentration following *in vitro* metabolism of AA in human and rat S9. The results, in accordance with *in vivo* studies [2,3], indicated that the oxidative transformation of AA to GA is faster in the rat than in human.

Any epoxide having at least one primary carbon could react with Cbl(I) to form an alkylcobalamin, as has been shown with butadiene epoxides [32,33], chloroprene epoxide [44], ethylene oxide [31] and styrene oxide [44] giving alkylcobalamins that are specific for each epoxide. In the present study, formation of GA-Cbl was used for the analysis of GA with ca. 100 times higher sensitivity than that obtained earlier in analysis of epoxides by using Cbl(I). The high sensitivity achieved by the Cbl(I) method in the analysis of GA in biological matrix opens for new applications in toxicological studies of GA and of other genotoxic epoxy metabolites. Amongst these one possible application would be screening for different epoxides present for instance in tissues and biofluids like blood.

Acknowledgments

The Swedish Cancer and Allergy Foundation and the Swedish Research Council Formas are acknowledged for financial support.

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