Cobalamin concentrations in fetal liver show gender differences: a result from using an HPLC-ICP-MS as an ultra-trace cobalt speciation method

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ABSTRACT

Maternal diet and lifestyle choices may affect placental transfer of cobalamin (Cbl) to the fetus. Fetal liver concentration of Cbl reflects nutritional status with regards to vitamin B12, at low concentration current Cbl measurement methods lack robustness. An analytical method based on enzymatic extraction with subsequent RP-HPLC separation and parallel ICP-MS and ESI-Orbitrap-MS to determine specifically Cbl species in liver samples of only 10-50 mg was developed using 14 pig livers. Subsequently 55 human fetal livers were analyzed. HPLC-ICP-MS analysis for cobalt (Co) and Cbl gave detection limits of 0.18 ng/g and 0.88 ng/g d.m. in liver samples respectively with a recovery of >95%. Total Co (Co_t) concentration did not reflect the amount of Cbl or vitamin B12 in the liver. Co as Cbl contributes only 45 +/- 15 % to Co_t. XRF mapping and μXANES analysis confirmed the occurrence on non-Cbl cobalt in the pig liver hot spots indicating particular Co. No correlations of total cobalt nor Cbl with fetal weight or weeks of gestation were found for the human fetal livers. Although no gender difference could be identified for total Co concentration, female livers were significantly higher in Cbl concentration (24.1 +/- 7.8 ng/g) than those from male fetuses (19.8 +/- 7.1 ng/g) ($p=0.04$). This HPLC-ICP-MS method was able to quantify total Co_t and Cbl in fetus liver and it was sensitive and precise enough to identify this gender difference.

INTRODUCTION

Vitamin B12 or cobalamin (Cbl) is an essential vitamin stored in the liver.[1] Cbl can occur in different molecular forms from which only two, methyl-cobalamin (Me-Cbl) and adenosylcobalamin (Ado-Cbl), are physiologically active. Me-Cbl is a cofactor for enzymes in the Carbon-1 metabolism, while Ado-Cbl is a cofactor for enzymes involved in 1,2 H-shifts and transfer of an electronegative group to the neighboring carbon atom. Although the majority of vitamin B12 is stored in the liver, the concentrations are at the ultra-trace level.[2] The methods routinely used to determine vitamin B12 status are either based on microbiological or immunoenzymatic determination of Cbl in serum and have been criticized for their overestimation or failure to determine low levels of Cbl and their lack of precision.[3,4] Analytical methods for Cbl determination based on chromatography coupled to ICP-MS or ESI-MS have been reported but so far mainly used for food-supplements.[5,6,7,8] An analytical method based on thermal acidic denaturation with liquid/liquid extraction of beef liver with subsequent RP-HPLC separation of the main four Cbl species with ESI-MS detection showed promising results for the determination of Cbl in liver samples.[9] The sensitivity and specificity of this method though needs improving due to the small size of tissue samples available from the human fetus and the extraction needs to be confirmed by using a complementary direct speciation method such as EXAFS and XANES.[10]

The wider aim of this study was to develop a method for Cbl quantitation which is robust but sensitive enough to detect low background levels in human fetal liver samples to determine maternal life-style influence on the fetal nutritional vitamin B12 status. This method was then applied to human fetal liver samples to identify whether vitamin B12 status varies with body weight, gestation age or gender.

This was achieved by the following objectives:

- To test whether it was possible to determine quantitatively the physiologically active forms of Cbl (Me-Cbl and Ado-Cbl) besides cyano-cobalamin (CN-Cbl), and hydroxyl-cobalamin (HO-Cbl) individually when spiked to liver in order to evaluate the full conversion of those active forms into CN-Cbl.

- To evaluate the quantitative extraction and determination of vitamin B12 and if possible the physiologically active forms of Me-Cbl and Ado-Cbl from pig liver samples using HPLC with parallel detection of using ICP-MS for Co and ESI-MS for the individual Cbl forms.
- To confirm whether extraction was altering the Cbl content by using a direct speciation method for which no extraction is necessary (by XRF (X-ray fluorescence) mapping with subsequent μXANES (x-ray absorption near edge spectroscopy).
- To apply the protocol to 55 human fetal livers and quantify the Cbl concentration and Co_t concentration in fetal livers and evaluate the results with regards to liver weight, sex and gestation age.

EXPERIMENTAL SECTION

Chemicals and Material

The different cobalamin (Cbl) standards, Methyl-cobalamin (Me-Cbl), Hydroxy-cobalamin (HO-Cbl), Adenosyl-cobalamin (Ado-Abl) and cyano-cobalamin (CN-Cbl) (98%, Sigma-Aldrich Germany) were dissolved in water with a concentration of 1 mg Co/mL and stored in the dark. The eluents for the HPLC were 0.1% formic acid (Analytical reagent grade, Fisher Scientific UK) in water (eluent A) and 0.1% formic acid in methanol (HPLC grade S, Rathburn UK) (eluent B). Co standards (High purity standards, UK) for calibration were prepared based on a stock solution of 1000 mg/L diluted with 1% HNO_3, conc. (supra pure, BDH UK). A Rh solution (Specpure, Alfa Aesar Germany) served as internal standard. For the sample preparation different organic solvents were used, including methanol (Laboratory reagent grade, Fisher Scientific UK), acetone (Laboratory reagent grade, Fisher Scientific UK), and additionally ultra-pure water (18 MΩ cm). For the liver extraction acetate buffer (pH 5) (acetic acid: extra pure, Sigma-Aldrich Germany), Papain (from Carica Papaya, Sigma Aldrich Germany), potassium cyanide (Fisher Scientific UK) and HCl (Laboratory reagent grade, Fisher Scientific UK) were used. Nitric acid conc and hydrogen peroxide (Laboratory reagent grade, Fisher Scientific UK) were used for the

microwave-assisted digestion of liver samples prior to total Co measurements by ICP-MS.

Pig Liver samples

For the method development 14 pig liver samples were used as a proxy for the human liver samples. The pig livers were bought at a local butcher in Aberdeen and stored at -20^{o}C before analysis.

Human fetal liver

The collection of fetal material was approved by the NHS Grampian Research Ethics Committees (REC 04/S0802/21). Women seeking elective, medical terminations of pregnancy were recruited with full written, informed consent by nurses working independently at Aberdeen Pregnancy Counseling Service. Only fetuses from normally-progressing pregnancies (determined by ultrasound scan), from women over 16 years of age with a good grasp of English and between 11-21 weeks of gestation, were collected.

Fetuses were transported to the laboratory within 30 minutes of delivery, weighed, crown-rump length recorded, and sexed. Livers were snap-frozen in liquid nitrogen and stored at -85°C. All morphological data were from the same study as published in Drake et al.[11] and are summarized in **Table 1.**

Table 1: morphological data for mothers and fetuses (mean ± SE)

	Females	**Males**
Maternal age (yrs)	25.0 ± 1.1	23.3 ± 1.2
Maternal BMI (m^2/kg)	24.6 ± 1.1	25.5 ± 0.9
N	25	30
Fetal weight (g)	122.4 ± 19.3	68.6 ± 11.1
Fetal crown-rump length (mm)	111.2 ± 6.3	95.1 ± 4.5
Fetal age (weeks of gestation)	15.7 ± 0.6	14.1 ± 0.3

Experiments and methods

Optimization of extraction method

All solutions were handled under dim light conditions. Cbl was extracted from homogenized freeze dried liver samples using a method modified from Rappazzo et al. (*12*). To 50 mg liver 10 µL potassium cyanide solution (1 % w/v), 500 µL buffer (50 mol L^{-1} sodium acetate pH 5.0) and varying amounts of papain were added. In the optimized final method 5 mg papain was added. The solutions were incubated at different temperatures and for different time periods and centrifuged after cooling in order to optimize the extraction efficiency with regards to total Cbl. The supernatant was stored at 4^{o}C in the dark until analysis.

Total cobalt (Co_t) determination

20-50 mg pig or human liver samples were weighed accurately in duplicate into a plastic centrifuge tube. Subsequently 2.0 mL HNO_3 were added and left to stand overnight at 25^{o}C. Hydrogen peroxide (0.5 mL) and 0.250 mL of 20 mg/kg rhodium as internal standard were added and the samples digested in a Mars 5 microwave oven (Matthews Inc, USA). Blanks as well as the listed CRMs were digested in every round of samples as well. Samples were cooled and diluted with deionised water to a final concentration of 2% (v/v) nitric acid. Cobalt was measured by high-resolution ICP-MS (Element 2, Thermo Fisher Scientific) at *m/z* 59 at low resolution (R = 300) in addition to m/z 103 for rhodium as the internal standard.

Cobalt speciation using HPLC-ICP-MS/ESI-MS

The separation and determination of the 4 cobalamin species was carried out by a reversed phase HPLC coupled to an ESI-MS and ICP-MS using a methanol gradient programme. The ESI-MS was used in positive FTMS-mode. The instrumental parameters are listed in **Table S1**. To allow quantification the reversed phase HPLC-ESI-MS was also linked to an ICP-MS. The HPLC flow was split before the UV-detector with a ratio of 3:1 (ESI-MS: ICP-MS), the continuous internal standard (Rh) used for ICP-MS was added via a T-piece before the ICP-MS nebulizer to correct for matrix changes. Parameters are listed in **Table S1** and further description of the split set up can be found by Bluemlein and co-workers.[13]

Synchrotron XRF mapping and μXANES speciation of Cobalt

Synchrotron based X-ray fluorescence (XRF) was used for mapping Co distribution in shredded freeze-dried pig liver samples. The samples were prepared as thin pressed pellets. Elemental maps were collected at beamline 20-ID (PNC/XOR) at the Advanced Photon Source (APS), Argonne National Laboratory.

The electron storage ring operated at 7 GeV. A nitrogen cooled Si(111) double crystal monochromator, calibrated using a cobalt metal foil, was used to generate the X-ray beam. The fluorescence signal was collected using a 13-element Ge detector (Canberra). Four maps of (1.5 x 1.5mm) were obtained by rastering the sample through the 9700 eV beam of 10 x 6 μm with a step size of 20 μm and an integration time of 0.3 s/step. The elemental mapping of trace levels of Co in a high Fe matrix (hemoglobin) is challenging due to the large overlap between the Fe Kβ emission line (7,059 eV) and the Co Kα emission line (6,915 eV). Therefore, a script was developed to subtract the contribution of the Fe Kβ signal from the sum of the Fe Kβ plus Co Kα signal based on the known ratio of Fe Kβ relative to the collected Fe Kα signal. Areas on the maps, corrected for Fe interference, showing accumulation of Co were investigated using μXANES in order to confirm the identification of Co and assess its speciation. Three scans per point of interest were collected, averaged and normalized using Athena.[14] The spectra obtained were compared to cobalt standards of vitamin B12 (CN-Cbl), coenzyme B12 (Ado-Cbl), methylcobalamin (Me-Cbl), hydroxycobalamin (HO-Cbl) and also Co^{+I} and Co^{+II} salts.

Quality controls and statistics

Blanks as well as CRMs were measured with every batch of the liver digests for total hepatic Co analysis. Certified standard reference materials (NIST RM8415, NRC TORT-2) were used to check reproducibility and accuracy, with both better than +/- 5 %. Spiking experiments into the liver sample of Cbl-species were performed to evaluate the integrity of the Cbl species and the accuracy and precision of the Cbl-determination.

Statistical analyses of data were performed using JMP 9.0.2 software (Thomas Learning, London, UK). For method development ANOVA two way tests were performed. For the human liver samples the normality of data distribution was tested with the Shapiro-Wilk test and non-normally distributed data were log-transformed

and re-checked for normality prior to analysis by ANOVA and Tukey-Kramer HSD and *t*-tests, where data were not normalized, or the variances remained unequal, non-parametric tests were performed (Wilcoxon Test).

Safety

Work with cyanide poses an extra level of risk, which needs to be assessed before starting to work. Especially cyanide should not be poured in acidic solution below pH 5 to prevent the generation of volatile HCN.

RESULTS AND DISCUSSION

Separation and detection

A mixture of the 4 Cbl standards in water was measured with the HPLC-ICP-MS. The 4 Cbl species were baseline separated on the C8 column with a methanol gradient. All were well retarded and separated within 14 min and were detected by their Co signal on m/z 59 by ICP-MS and simultaneously by their molecular peaks $[M+1]^+$ and $[M+1]^{2+}$ by ESI-MS (**Figure 1a-b**). It can be seen that the ICP-MS Co response did not change significantly during the chromatographic run although a gradient programme was used (**Figure S1**). This behavior is in contrast to what has been observed for arsenic or sulphur,[15] because Co does not benefit from the carbon enhancement effect since it is already fully ionized in the plasma. The response factor for the Orbitrap varied considerably depending on species as indicated in the different peak heights (**Figure 1a**). Using the elemental calibration (**Figure S1a-b**) the amount of cobalt can be calculated for each species using the ICP-MS signal, whereas when solely the ESI-MS is used then for every Cbl an individual calibration curve is required. For quantification an external calibration was used with Co element standards (Co^{2+}) from 1 to 100 µg Co/L using the ICP-MS signal. The calculated detection limit for aqueous solutions is about 0.05 µg Co/L based on 3 times standard deviation of the background noise. This is more sensitive than the methods listed in a recent review.[2]

Stability of the standards over time

In order to assess the stability of cobalamins (objective 1), a comparison between freshly prepared and stored (frozen) solutions was performed (**Figure 1b and 1c).**

When the standards were stored for more than a day in a freezer, species transformation took place. CN-Cbl was stable, while Me-Cbl and Ado-Cbl showed only recoveries of 5.2 % and 10.6 % respectively. The overall recovery was however around 90%, since the unstable species transformed to HO-Cbl, which almost tripled in concentration (280%). This confirms the recent study of Szterk et al. (*9*) who found that these transformations may be through oxidation in air and UV radiation, which result in the conversion of all physiologically important species to HO-Cbl. Hence, the samples need to be measured immediately after extraction.

Stability of cobalamin species in different extractant solutions

To extract the different Cbl species from liver, they have to be liberated from their transport-proteins and transferred, unchanged, into the extract and the majority of the matrix should be removed. Several possibilities were tested for their influence on Cbl-speciation by spiking experiments using pig liver in order to address objective 1 and 2. Treatment of Cbl-standards with papaine, diluted nitric acid, methanol or acetone resulted in species transformation. When all Cbl species were heated in acetone or methanol in order to precipitate all proteins all Cbl species eluted in the void and did not show the corrin-ring moiety (evident through missing $[M+H]^+$ data), this means transformation to unbound polar Co species took place (early eluting Co compounds close to the void volume). Hot water extraction at 50^{o}C of the pig liver with subsequent measurement of Co speciation showed that part of Ado-Cbl in the pig liver was stable during this extraction (**Figure S2**). The majority of Co eluted however in the void (**Figure S3**). Spiking of all four Cbl species into the pig liver sample revealed also that the Me-Cbl and Ado-Cbl transferred mainly to HO-Cbl rather than unbound not retarded Co. Hence, the reliable quantification of the two bioactive Cbl species was not possible. Since the aim is to have a sensitive method for total Cbl in contrast to any non-Cbl (inorganic Co), we tested the CN-Cbl method when all the Cbl species should be converted quantitatively as CN-Cbl (**Figure S4**). This method was originally developed for the extraction of Cbl species from serum (*2*). Quantitative conversion was tested by spiking pig liver with about 4 µg Co/g of all four Cbl species in triplicates, and study their stability in the KCN liver extracts.

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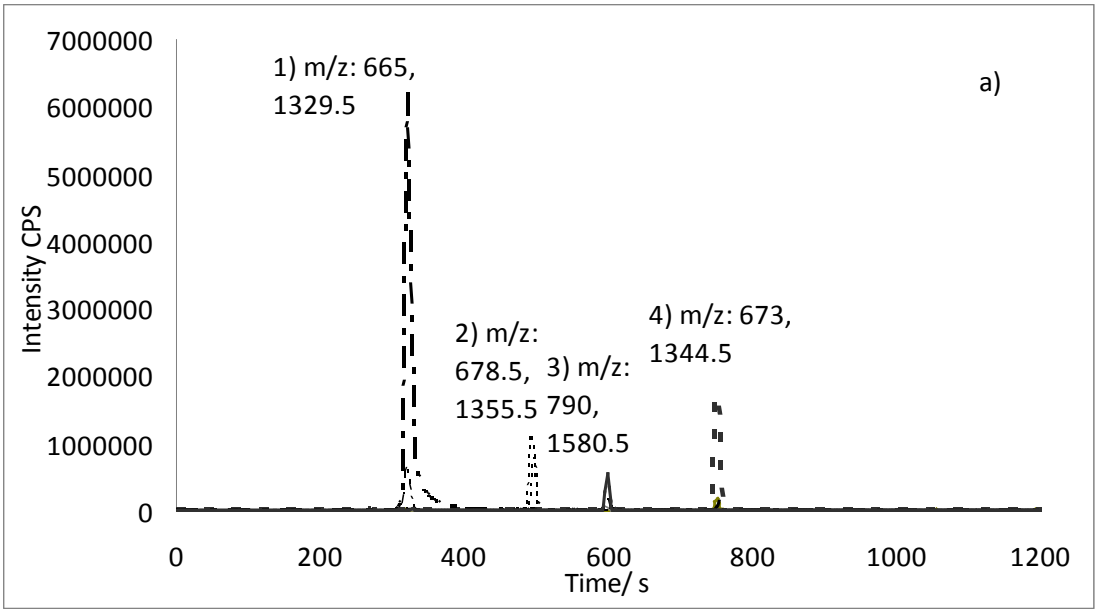
a)
1) m/z: 665, 1329.5
2) m/z: 678.5, 1355.5
3) m/z: 790, 1580.5
4) m/z: 673, 1344.5
Intensity CPS
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
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200
400
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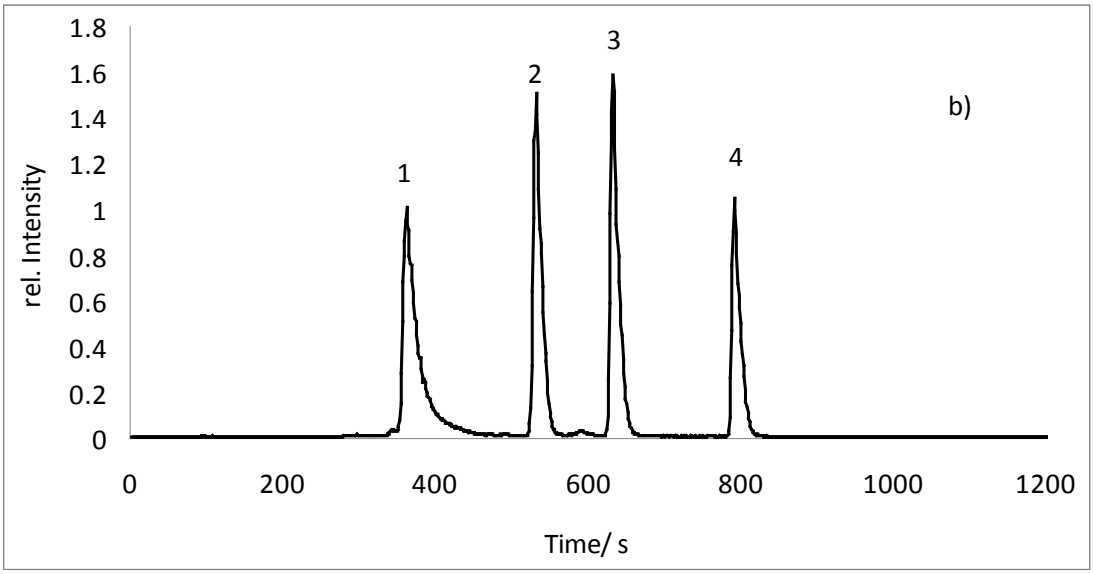
1200
Time/ s


b)
rel. Intensity
1.8
1.6
1.4
1.2
1
0.8
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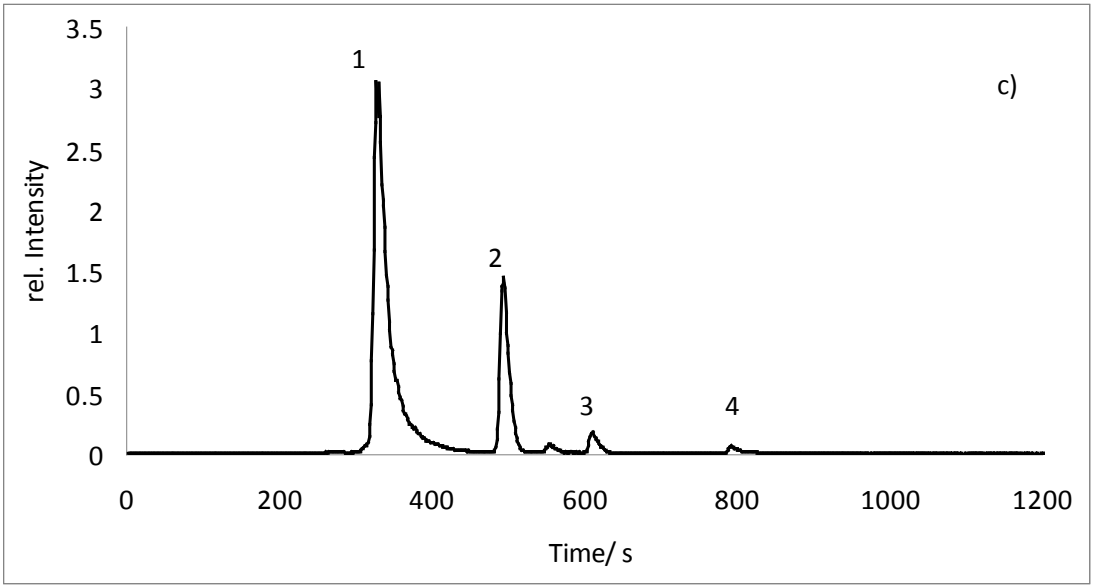
c)
rel. Intensity
3.5
3
2.5
2
1.5
1
0.5
0
1
2
3
4
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200
400
600
800
1000
1200
Time/ s

272

Figure 1a-c: Chromatographic separation of four different cobalamin species using reverse-phase HPLC detected on their $(M+H)^+$ and $(M+2H)^{2+}$ by ESI-Orbitrap MS (a) and simultaneously on m/z 59 for cobalt by ICP-MS (b) within 13 minutes. Peaks are 1) HO-Cbl (m/z 665, 1329.5), 2) CN-Cbl (m/z 678.5, 1355.5), 3) Ado-Cbl (m/z 790, 1580.5), 4) Me-Cbl (m/z 673, 1344.5). The degradation of a standard under oxygenated conditions at room temperature is shown in c).

To minimize the risk of underestimation of Cbl in liver samples Ado-Cbl and Me-Cbl were not determined as their individual species but rather than quantitatively converted to CN-Cbl by the addition of sufficient cyanide. Additionally only one peak needs to be integrated which would make the SOP easier and lowers the error. When extracted with the aid of cyanide the resulting chromatogram shows only two Co peak, one for unbound early eluting Co and one for CN-Cbl as illustrated in **Figure 2**. None of the Cbl species seems to loose Co under the tested conditions. The spiked pig liver did not show an increase in the early eluting (unbound) Co, and only one prominent Co peak, that of CN-Cbl (**Figure 2**). The column recovery was around 95 %. The conversion of all spiked Cbl species to CN-Cbl was quantitative (94 +/- 2%; n=3) which render this method to be accurate. Although the recovery of the spiked Cbl species was quantitative the extraction of Co_t was not (**Figure S5**). The Co_t concentration of the unspiked pig liver was 57 +/- 4.7 ng Co g^{-1} d.m. (**Figure S6**) while the Co_t determined in the extract was only 32 +/- 1.7 ng Co g^{-1} d.m., hence the extraction efficiency of Co_t was only 56 %. Although the nature of the unaccounted Co species was unknown the extraction method for Cbl was further optimized in order to prevent potential loss of Cbl species in the liver samples by varying the papain amount, the temperature and incubation time. The optimized extraction efficiency was 71 ± 28 % (n=4) of cobalt using between 10-50 mg liver 5 mg papain with 3 h incubation at 37°C. (**Figure S5**). Although the spiked Cbl gave an excellent precision of +/-2%, the precision of the intrinsic Co_t in the liver was higher (+/- 40 %) at the level of 4 μg Co as Cbl/g. This indicate that the liver samples were not homogeneous with regards to the Co_t when only 10-50 mg samples were taken. Hence, the homogeneity of the sample was investigated by using the XRF mapping (objective 3).

Using 3 times the standard deviation (SD) of the blank level, and a sample mass of 50 mg d.m., the method for has a detection limit of 0.18 ng/g d.m. for Co_t, while for the speciation for total Cbl a lower detection limit of 0.88 ng Co/g d.m. was established. A practical lower limit of quantification (10 times the SD of the blank) is therefore

about 3 ng Co as Cbl/g d.m. liver. This means that the described analytical method was capable to detect between 10-50 pg Co as Cbl (depending on the weight of the sample). This is superior to all so far described methods.[2,9,12] This should be lower than the expected levels of those analytes in human fetal liver.

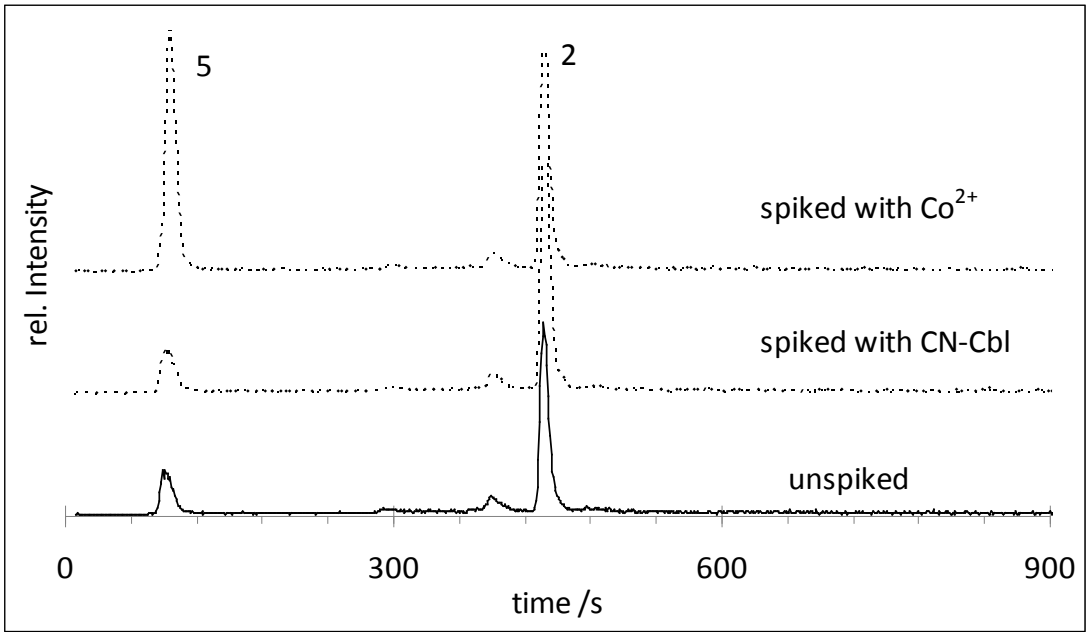

Figure 2: HPLC-ICP-MS chromatogram shows a pig liver extract using cyanide of an unspiked and a CN-Cbl (peak 2) and Co^{2+} as nitrate spiked extracts (peak 5) gives the inorganic cobalt in the extract, while peak 2 shows CN-Cbl.

Cobalt speciation in pig liver

The optimized method was applied to 14 pig liver samples. The amount of sample used during this experiment was kept to below 50 mg per sample in order to evaluate the suitability of the method for human fetal samples. Co_t varied significantly over almost one order of magnitude (18-145 ng Co/g *d.m.*). The extraction efficiency was measured for a subset (2 samples, n=3) gave 89 ± 14 % with an outlier of only 43 % (**Figure S7**). All samples (n=14) were extracted for speciation analysis with the optimized method. The extractable Co concentration ranged from 18 – 50 ng Co/g d.m. Although the extraction efficiency was nearly quantitative, only a fraction of total Co was in the form of Cbl measured as CN-Cbl (**Figure S8**). The Cbl fraction accounted for 45 ± 15 % of Co_t, while non-specified unbound cobalt was nearly 55 %

with little unaccounted non-extractable Co. While Co_t concentration was highly variable the Cbl-concentration was remarkable constant with 23 ± 8 ng Co/g *d.m* (n=14). Interesting is the variation between the different liver samples; the liver sample CC-5 contained around 68 % of Co as Cbl in the extracted material, while L1F had only 25 %. This variability has been seen in beef livers before.[1] Considering only the extractable Co, Cbl shows a logarithmic trend when related to extractable Co (**Figure 3**). That indicates that high total Co concentrations in pig livers might not be the result of vitamin B12 accumulation but rather of Co which is not bound as Cbl. This contradicts that the amount of Vitamin B12 linearly depends on the amount of Co_t in liver reported elsewhere (*1*). Hence, the Cbl concentration cannot be estimated from the total Co concentration in pig liver. The amount of Cbl needs to be measured directly in order to give a reliable account of the vitamin B12 concentration.

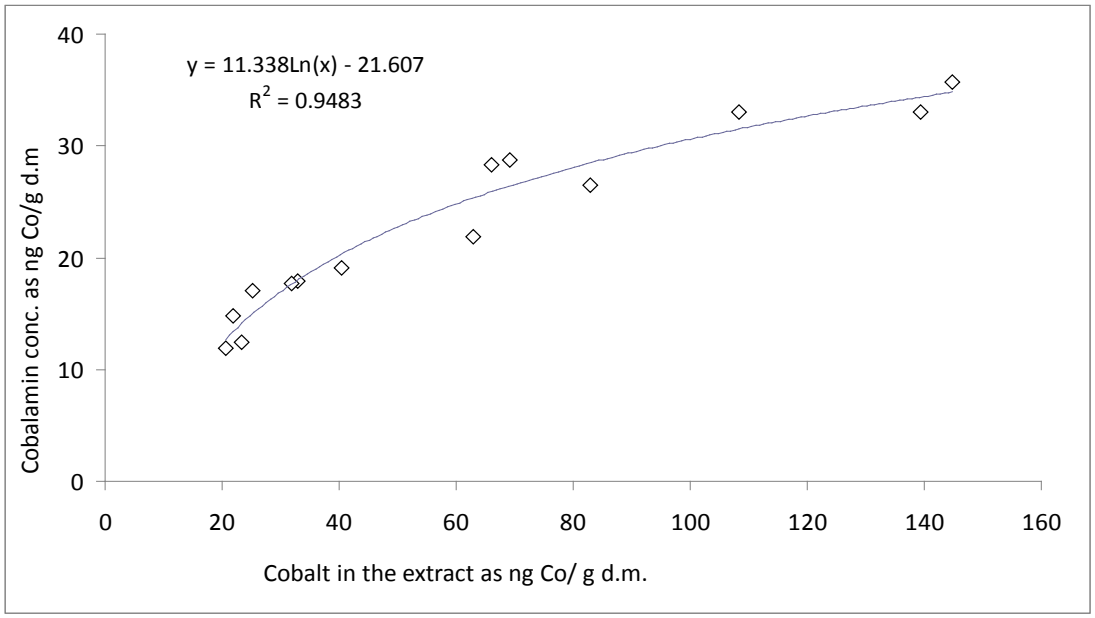

Figure 3: Correlation cobalamin expressed as cobalt versus the cobalt concentration in the extract of 14 pig liver samples.

In general, the results for the spiked pig liver suggest that full conversion of all Cbl species to CN-Cbl was achieved. The separation of the Co species has been shown to be robust (retention times did not vary more than 0.1 min) throughout the analysis. Although pooled samples showed good reproducibility in their Co concentration (approx.. 5% **Figure S5**), subsamples taken from individual livers showed

considerable variability (**Figure S6 and S7**). This may suggest that cobalt is heterogeneously distributed throughout the pig liver especially if only a small sample is taken, which would be unexpected for physiologically regulated Cbl.

To shed more light on the heterogeneity of Co and Cbl in the liver and whether Cbl species transformation had taken place during the sample preparation, i.e. the release of cobalt from the corrin ring, XRF mapping of the pig liver and subsequent μXANES was used for unspiked pig liver samples which showed qualitatively the occurrence of Ado-Cbl. The challenges to overcome were first the low concentration of cobalt < 0.1 mg/kg and the interference of the Fe Kβ fluorescence, which overlaps with Co Kα. Therefore Fe and Co were measured simultaneously and every pixel was corrected using Co Kα - Fe Kα/Kβ resulting in a cobalt specific map of the liver sample. The results clearly indicated the presence of Co in small hotspots (approximately 10 to 30 μm in size) throughout the samples (**Figure 4 and S9)**. The XANES spectra of the cobalt hotspots seems similar to inorganic Co^{+II} and Co^{+III} compounds and distinctively different from the XANES spectra of cobalamin standards characterized by a double feature in the main absorption peaks ($Co^{+II/III}$). Although the nature of these hotspots are unknown, it is not inconceivable that these hot spots are the result of absorbed cobalt containing particles. This explains would explain the heterogeneity of Co_t but the homogenous distribution of Cbl. Due to the above mentioned Fe interferences in these samples, a homogeneously low distribution of cobalamin in the sample would not be detected either by XRF or μXANES. However, XANES and XRF analysis suggests that the majority of Co in the pig livers were not in the form of Cbl but rather in the form on unbound Co^{+II}. Therefore, this confirmed the relatively low extraction efficiency of Co_t (70-80%) combined with the high recovery of spiked Cbl species. Hence, the described methodology with a low limit of detection (< 1 ng Co as Cbl/g d.m.) and its precision of < 5% and its accuracy of 94% it was suited to use for the determination of Cbl in fetal liver samples.

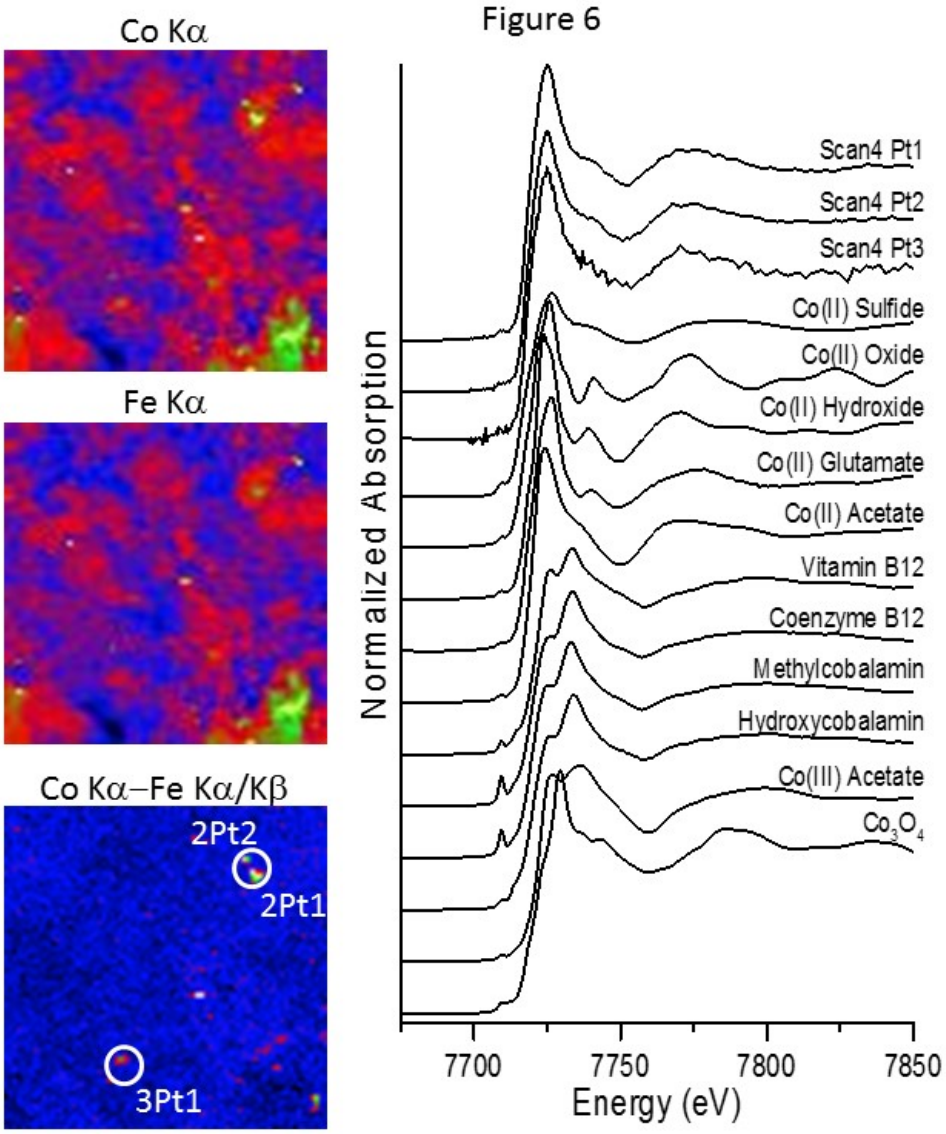

Figure 4a-b: 2 D cobalt map (1.5 x 1.5 mm)(a) from a shredded pig liver paste (resolution of about 20 μm with the μXANES spectra taken at the hotspots (b) in comparison to the XANES spectra of four different cobalamin standards.

Human fetal liver samples

Co_t in human fetal samples was analyzed in duplicate and showed a high variability (25 to 190 ng Co/g d.m., detection limit 2 ng/g *d.m.* **Figure 5**). All livers had Co_t and

Cbl concentrations above the detection limits. The first results of the study has been published partly by Drake et al.[11] with regards to lifestyle influence on the Cbl concentration in the fetal livers without describing the analytical method in detail. Here, we describe the analytical method capable of measuring Cbl with high sensitivity and precision and subsequent aspects of the study which enabled us to look at a part of the study which was previously not described.

There was no correlation between weight and the Co_t concentration neither was a significant gender difference found (unpaired two-way ANOVA, $p=0.082$). The Co_t concentrations were comparable with those reported in by Caldas and Dorea.[1] When, however, the Cbl concentration was measured, a significant gender difference could be established (two-way ANOVA, $p<0.05$). The Cbl concentration in the female liver of non-smoking mothers was 643 ± 48 ng Vitamin B12/g dm, whereas male fetal liver of non-smoking mothers contained 497 ± 51 ng Vitamin B12/g. The reason why there is a gender difference is still unclear and how the C1 metabolism of the fetus is influenced when the mother smokes has been discussed elsewhere.[11] The data also indicate that the vitamin B12 concentration correlates linearly with the Co_t concentration in the liver of the fetuses independent on the gender ($P<0.001$) (**Figure 5**).However, even if the correlation is significant the variability was still very large within the data set and a precise measurement of Cbl needs to rely on direct measurement rather than interpolation from total Co (**Figure 5**).

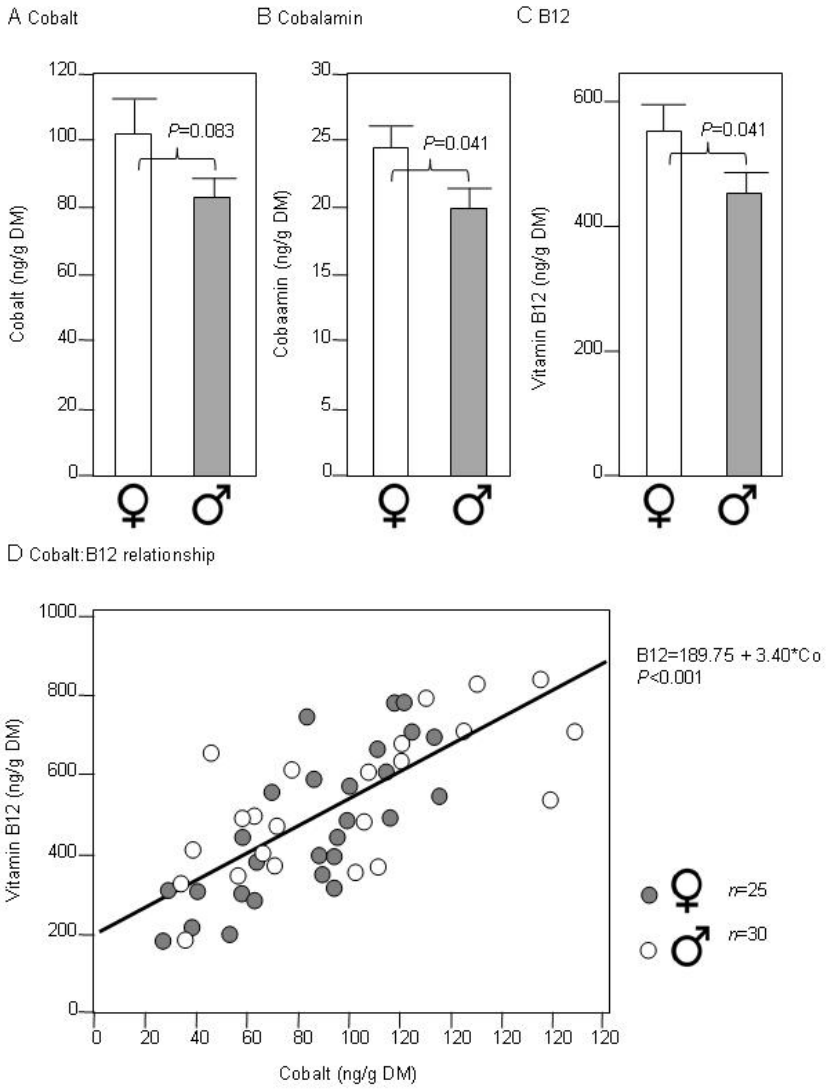

Figure 5: Co_t (A) and Cbl concentration (expressed as ng Co/g liver (B) and as ng Cbl/ g liver (C) in human fetal livers show a significant lower cobalamin level for female fetal livers. The p-values given are based on ANOVA unpaired two way tests. (D) shows the correlation of vitamin B12 and Co_t.

CONCLUSION

The method to determine cobalamin in liver samples described here is sensitive enough to determine background levels of unbound and bound cobalt in fetal liver samples. Although, we were unable to determine the individual physiologically active

forms of Cbl (Me-Abl and Ado-Cbl), all forms of Cbl could be transformed into CN-Cbl and determined quantitatively in liver samples with an accuracy of around 94 % and a precision of +/- 5 %. A significant amount of Co is in a non-characterized form in the extract, which however is not an artefact of the extraction method and a degradation product of Cbl species. Not only is Co_t not representing the amount of Cbl in the liver samples, the analyte is subject to large variability through the accumulation of inorganic Co, which seem to point to particulate Co. The nature of this uncharacterized cobalt needs to be studied in the future.

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Figure 1