Title

Comparison of GC/EI-MS and CF-IRMS in expiratory breath $^{13}CO_2$ stable isotope dilution.

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Abstract

We investigated the potential of a GC/Ei-MS method as an alternative to IRMS in breath $^{13}CO_2$ / $^{12}CO_2$ analysis.

Introduction

Investigations on protein metabolism in humans can be performed with the isotope dilution technique[1]. This technique included priming with $NaH^{13}CO_3$ uses infusions of L-[1-^{13}C]Leucine (measured as ^{13}C-Ketoisocaproic acid in blood samples) combined with identification of enrichments of $^{13}CO_2$ in expired air samples. The enrichments of L-[1-^{13}C]Leucine and after analogous infusion of [6,6-$^{2}H_2$]glucose can be quantified by GC/MS-PCI[2] and GC/MS-NCI[3] because of relatively high differences between baseline contents and steady state isotope ratios (1-4%)[4]. Usually the isotope ratio mass spectrometry (IRMS) is employed[5] to identify changes in the $^{13}CO_2$ / $^{12}CO_2$ ratio in expiratory breath samples. Its accuracy predestines the method because of the extremely small differences (about 0.005 atom%) in ^{13}C content in carbon dioxide[5] caused by ^{13}C-enriched infusions. Earlier attempts

demonstrate the difficulties when other methods are used[6]. Nevertheless we tried to apply our gas chromatograph coupled to a mass selective detector (GC/MS-Ei) for the purpose of $^{13}CO_2/^{12}CO_2$ relation analysis.

Experimental

The stable isotope-enriched substance L-[1-^{13}C]Leucine was purchased from Euroiso-top (Saarbrücken, Germany). Expiratory air was sampled in 2 liter plastic bags and transferred into 5 ml Vacutainers (Bekton Dickinson, Plymouth, UK) with 50 ml glass syringes (popper & sons, New York, USA) to be sent to ISO Analytical Ltd. (Sandbach, Chesire, UK) for IRMS analysis with an Europa Scientific GSL/Geo 20/20. Sample preparation for GC/MS was also done by filling 50 ml glas syringes with expiratory air out of the plastic bags. To get storable samples 2 ml autosampler crimp vials (CZT, Kriftel, Germany) were overfilled about 20 times by the glass syringes and immediately sealed.

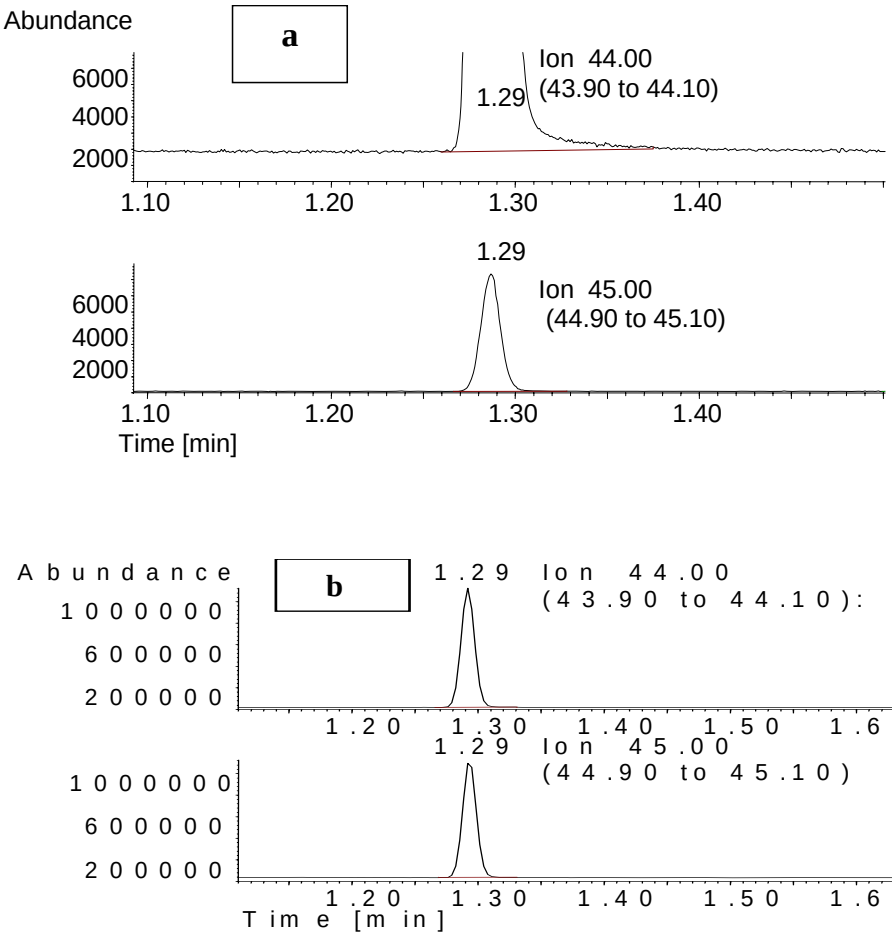
Statistical analysis was performed with GPOWER[7] and SPSS for Windows, version 10.0 (SPSS Inc., Chicago, IL, USA).

Chromatography

Our chromatographic equipment was an Agilent 6890plus with an Agilent 5973 mass selective detector (Agilent, Waldbronn, Germany) in electronic ionisation (Ei) – single ion mode (sim) and a widebore GS-GASPRO column (30m*0.32mm) also from Agilent. A gas volume of 2.5µl was injected by an A200S autosampler (CTC-Analytics, Zwingen, Switzerland) with a 10µl Hamilton (Darmstadt, Germany) syringe into the SSL injector at 265°C and a split ratio of 50:1. The oven was kept constant at 80°C with carrier gas 99.999 % pure helium (Linde, Hoellriegelskreut, Germany) at a pressure of 134.4 kPa and the transfer line at 300°C. Total run time was 1.5 min and the retention time for CO_2 was 1.29 min detected once at 44.0 m/z and 9 times at 45.0 m/z with a dwell time of 10 ms each. The

estimated limit of detection (signal to noise ratio about 3 to 1) was about 70 times lower than the natural $^{13}CO_2$ concentration in expiratory breath (see figure 1) even without the ninefold amplification. Because of the relatively high and almost constant concentrations of $^{13}CO_2$ and $^{12}CO_2$ we did not identify the lower limit of detection (LLOD) exactly.

Figure 1: Chromatographic examples for $^{13}CO_2$ and $^{12}CO_2$ peaks from air without (a) and with (b) ninefold amplification of the $^{13}CO_2$ (45 m/z) signal



Results

First attempts in air isotope analysis without separation of the gases N_2, O_2, CO_2 failed because of mass interactions from the main air components overlapped the trace amounts of

$^{13}CO_2$. An improvement of the situation could easily be achieved by assembling an adequate gas separation column into the GC. An other problem was the unfavourable Peak area ratio of the CO_2 peaks of about 1:88 ($^{13}CO_2$: $^{12}CO_2$; natural ^{13}C content in CO_2 is about 1.12%, figure 1a). Peaks of comparable area for $^{13}CO_2$ and $^{12}CO_2$ could be achieved by adapting the scan ratio from 1:1 (44m/z : 45m/z) to 1:9 (44m/z : 45m/z) (figure 1b). With these advanced conditions we analysed a couple of end expiratory breath samples. The samples were kept in usual glass vials and were air tight crimped prior to analysis. Repeated injections showed good reproducibility (Table 1) without significant changes during multiple penetration of the septum.

Table 1: Preliminary tests of reproducibility with repeated air injections

Series	Number of injections	Mean Area ratio (44.0m/z : 45.0 m/z))	SD	Range	Min	Max
1	16	1.0015472	0.004937	0.01625	0.99368	1.00993
2	16	1.0034810	0.004925	0.01951	0.99142	1.1093

Encouraged from these data we analysed air probes from a patient included in a study [8] (Patient 1) to identify base level and averaged steady state level of $^{13}CO_2$ according to our standardized procedure (Table 2). The mean area% of 45m/z in series 0 (baseline) was significantly lower ($p<0.05$; one way ANOVA with post-hoc Dunnett-T test) than in all following series (150-180 min, steady state, for detailed information of the proceeding see [8]). The data of table 2 showed in an a priori analysis based on the results in table 1 with the "Gpower" software[7] that a total sample size of 8 is sufficient for identifying significant differences in means (alpha = 0.05, power = 0.90)

Table 2: Preliminary tests of air-probes from patient 1. Series 0 was collected just before infusion start of $NaH^{13}CO_3$. Series 150-180 were taken from 150 min until 180 min after start of infusion. The difference of means to series 0 is significant in all following series as indicated by the asterisk * ($p<0.05$).

Series	Number of injections	Mean Area % of (45 m/z) to total (44m/z + 45m/z)	SD	Range	Min	Max
0	8	49.419900	0.0606	0.218	49.3088	49.5268
150*	8	49.605212	0.0727	0.220	49.4629	49.6836
160*	8	49.569059	0.0788	0.230	49.4672	49.6979
170*	8	49.573879	0.0607	0.185	49.5004	49.6853
180*	8	49.591378	0.0641	0.193	49.4727	49.6660

Discussion

Multiple preparations and analysis of samples are generally used to approve the efficiency in accurate determination of Xenobiotics in biological material. Double determination of pharmaceutics in patients blood is standard as well as the coverage of an ELISA result by a second probe. We tried to carry the number of injections to the extreme by exhausting the automatic injection system with 8 injections per probe. The reproducability within one series (Injections out of one vial) is adequate (fig 2, table 1 and 2). We could qualitatively identify every enrichment of $^{13}CO_2$ in our patients samples. The absolute values of the $^{13}CO_2$ rise avoided identification by GC/MS because of the artificial multiplying of the $^{13}CO_2$ signal along with the MS-detection without standard. For the comparison of $^{13}CO_2$ enrichment

satisfy relative changes. As documented in the weak correlation in figure 3 the relationship between absolute delta values by IRMS and area percent in GC/MS $^{13}CO2/^{12}CO2$ analysis are inadequate. Relative differences (averaged steady state to baseline from every patient) are to be fixed almost acceptable (fig 4).

Figure 3: Comparison of absolute delta ranges from IRMS versus changes in area % of $^{13}CO_2$ to total CO_2 in GC/MS runs from 54 identical patients expiratory gas samples. (data from 13 patients prae and post op, 1 patient only post op because of leaky vials, means of steady state (4 times 8 injections) and baseline (8 injections))

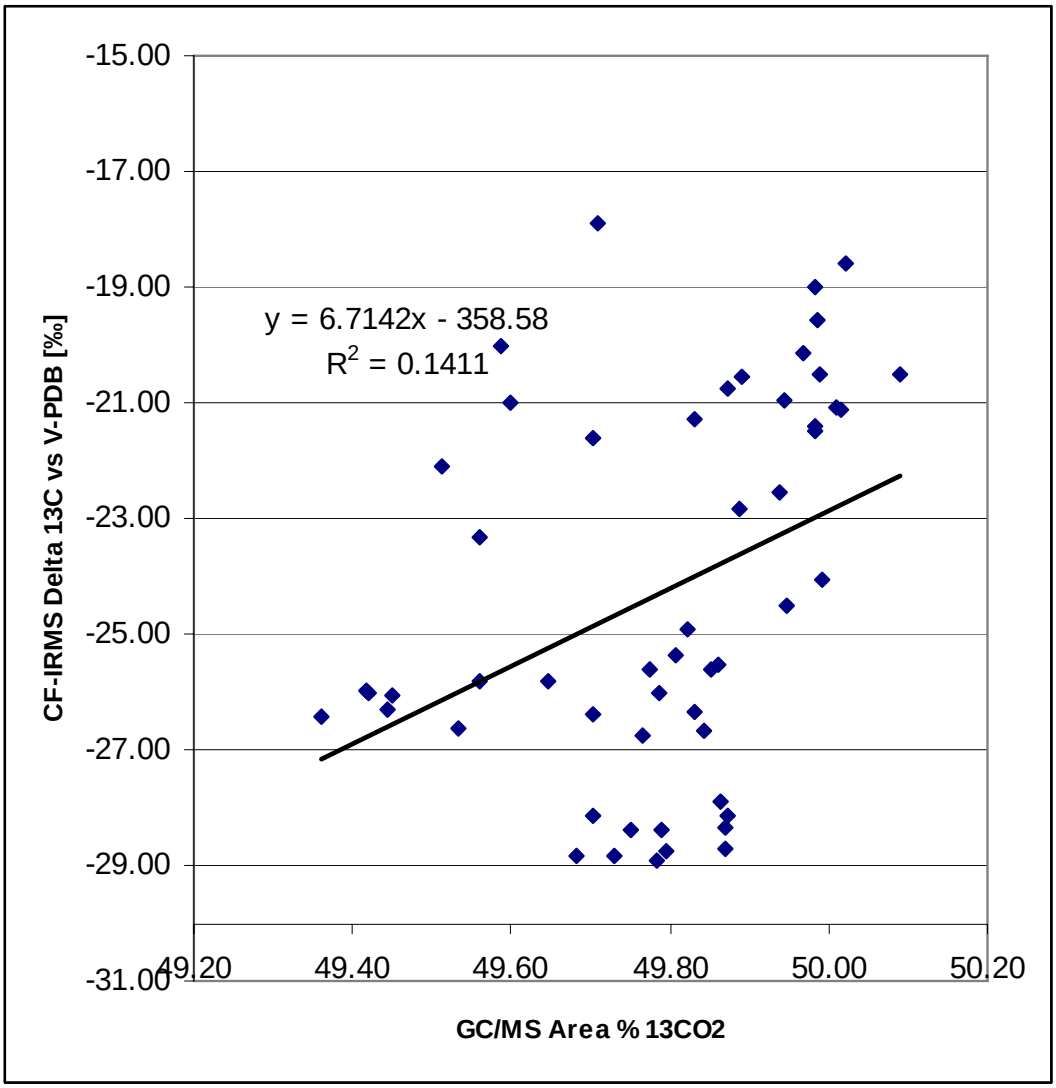
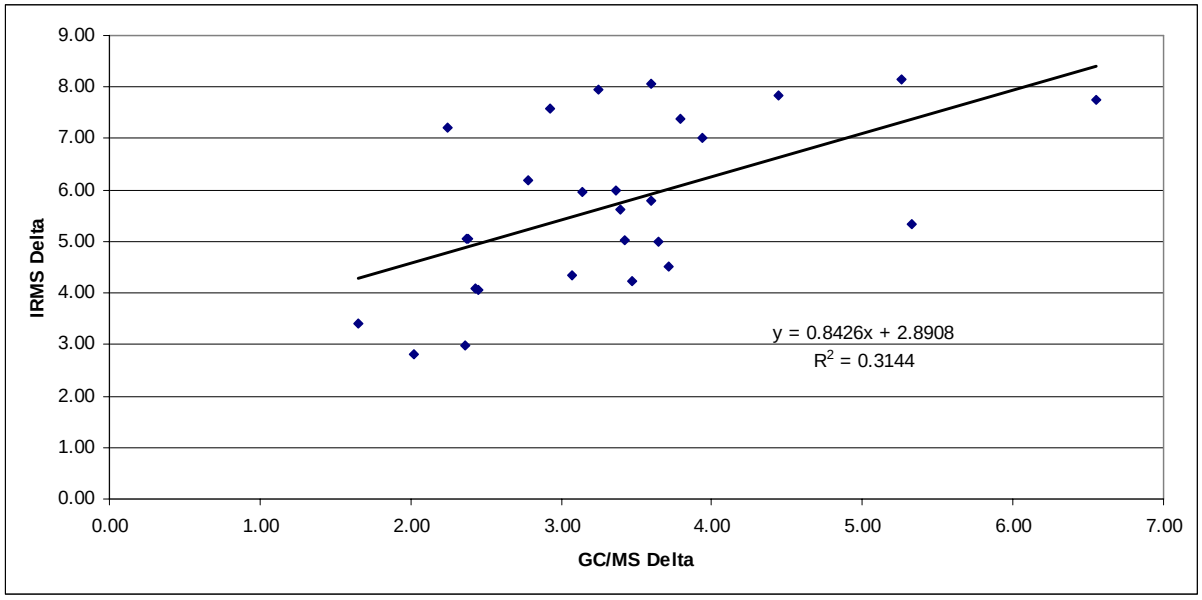


Figure 4: Comparison of Delta values at steady state referring to the baseline before ^{13}C priming. Data from 13 patients prae and post OP, 1 patient only post OP, means of steady state (4 times 8 injections minus mean of baseline (8 injections) (IRMS Delta: Difference of delta ^{13}C versus V-PDB at steady state minus delta ^{13}C versus V-PDB at baseline; GC/MS delta: calculated by the formula ((area% ^{13}C at steady state/area% ^{13}C at baseline)-1*1000)



Nevertheless the stability and reproducability of data obtained from the CF-IRMS is the unbeaten state of the art as documented by a delta ^{13}C versus V-PDB of expected –33.68 ‰ and measured –33.64 ‰ (s.d 0.04 ‰) from a reference CO_2 (IA-CO_2-3) (International Atomic Energy Agency, Vienna, Austria). Corresponding ^{13}C atom% show a 46.5 times smaller CV than two series of CG/MS area% analysis (table 3). Aside the quality of the MS systems reproducibility of the sample preparation has to be taken into account.

Table 3: Comparison of the CO_2 QC- Data from CF-IRMS and GC-MS. IRMS data are from the reference gas and GC/MS data are from expiratory breath.

Series	Number of injections	^{13}C Atom%	Mean Area % of (45 m/z) of total (44m/z + 45m/z)	SD	CV [%]	CV mean ratio
IRMS-1	13	1.074238		7.0755E-5	0.00658	1
IRMS-2	9	1.074208		4.3278E-5	0.00402	
GC/MS-1	16		49.9616	0.1232	0.2465	46.5
GC/MS-2	16		49.9134	0.1230	0.2464	

In conclusion multiple injection technique with a GC/MS system is not capable to displace CF-IRMS but allows an effective first glance on ^{13}C isotope enrichment in breath analysis.

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