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# Xenon detection in human blood: analytical validation by accuracy profile and identification of critical storage parameters

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# Xenon detection in human blood: analytical validation by accuracy profile and identification of critical storage parameters


## Abstract

Xenon is a rare, mostly inert, noble gas that has applications in a wide range of fields, including medicine. Xenon acts on the human body as a useful organ-protective and anesthetic agent and has also been previously studied for potential applications in fields such as optics, aerospace and medical imaging. Recently, it was discovered that xenon can boost erythropoietin production, and it has been used as a performance-enhancing agent in international sports competitions such as the Sochi Olympic Games. Therefore, screening methods to detect the misuse of xenon by analysis of biological samples and to monitor anesthesia kinetics and efficiency are being investigated. The aim of this study was to develop and validate an analytical method to detect xenon in blood samples using gas chromatography coupled to tandem mass spectrometry (GC-MS/MS).

Preliminary studies were conducted to determine the best parameters for chromatography and mass spectrometry for xenon. The analysis was performed using the multiple reaction monitoring (MRM) mode using the transitions *m/*z 129→129, 131→131 for xenon and 84→84, 86→86 for krypton, which was chosen as the internal standard. The LOD of GC-MS/MS was found to be 52 pmol on-column. Calibration lines and controls were made to obtain an accuracy profile at a range of 2.08–104 nmol with a β-expectation tolerance interval set at 80% and the acceptability limit set at ±30%. From the accuracy profile, the LOQ of 15 nmol on-column for the range of

2.08–104 nmol was obtained. The method was validated according to the guidelines of the French Society of Pharmaceutical Sciences and Techniques.

The detection method was finally validated using blood from test persons subjected to a 15% or 30% xenon mixture with pure oxygen and air for 45 minutes. Even though the probes were already used for other projects, it was still possible to detect xenon.

**Keywords:** xenon; anaesthesia; anti-doping; accuracy profile; blood analysis; GC-MS/MS

## Introduction

Xenon is a noble gas that was initially found in very small amounts (0.087 ppm) in the composition of liquid air in 1898, and this element possesses some interesting physiological properties.[1] In 1939, it was discovered that xenon can act as an anesthetic agent, and in 1951, the first surgery with xenon was performed.[2,3] Since then, much research has been conducted on this gas to better understand its mechanism of action in the body.[4-7] In addition to its anesthetic properties, xenon has organ-protective properties that may be based on its interaction with the hypoxia-inducible factor HIF-1α.[8,9] When this factor is activated, it increases the synthesis of erythropoietin (EPO), a substance commonly used in doping.[10,11] For this reason, the World Anti-doping Agency (WADA) decided to ban the use of this substance in September 2014. The determination of xenon in human blood is therefore of practical interest for medical anesthesia and doping control.

The absorption, distribution, metabolism and excretion of xenon are very simple, but descriptions of these processes in the literature are scarce. According to a recent study on xenon elimination kinetics following brief exposure, xenon is inhaled through the lungs and reaches all of the compartments (i.e., lungs, brain, liver, muscle, tissues, and fat tissues). The gas is not metabolized, and once the patient stops inhaling the gas, it washes out quickly and is mainly excreted during two phases, which is typical for substances stored in multiple compartments, including anesthetics.[12] The first phase leads to xenon elimination of approximately 95%, which is exhaled in the first pass by the lungs and shows a half-life of 2.7 h.[13-15] The second phase, which is much slower and results from residual amounts of xenon, is expressed by a non-linear regression characterized by first-order kinetics and could be related to body fat content due to the solubility of xenon in fat matter.

Xenon gas is not metabolized by the body and is rapidly eliminated, and analysis of this gas requires analytical methods with high sensitivity because the xenon concentration measured in blood is usually in the nmol/mL range (ppb). However, the concentrations of xenon used for anesthesia are higher than those used for doping purposes and are easier to measure. For example, xenon can be detected in blood up to 30 h after storage and in urine until 40 h post-anaesthesia.[16,17] Consequently, gas chromatography coupled to mass spectrometry (GC-MS) and tandem mass spectrometry (GC-MS/MS) seem to be the best candidates for detection, and these methods have already been investigated.[16-18] To this end, MS/MS may be more efficient because even if no fragmentation occurs, the energy of collision can be applied to reduce background noise. As no cut-offs have been set for this substance, the first analytical step is to detect xenon in biological samples, and the next is to provide an estimation of the concentration even if the physiological significance remains unknown.

The aim of this study was to develop and validate an analytical method to detect xenon in biological samples. GC-MS/MS is the most appropriate method because it combines high separation power from GC with high selectivity from tandem mass spectrometry, which allows the background interference to be minimized and improves both the selectivity and sensitivity. The validation step involves demonstrating that the analytical method is appropriate, reliable and precise for this potential use. Subsequently, this method was applied to biological samples obtained from study subjects after xenon inhalation to determine whether it was possible to detect this substance in the samples.

**Experimental design**

Materials

Xenon (purity, 4.0) and krypton (purity, 4.0) were purchased from Carbagas AG (Lausanne, Switzerland) and PanGas AG (Dagmersellen, Switzerland), respectively. Bovine blood samples for blanks were obtained from the Forensic Toxicology and Chemistry Unit at the University Center of Legal Medicine (Lausanne, Switzerland).

Authentic blood samples were obtained from three volunteers from the University Hospital RWTH Aachen (Germany) as part of a study on male and Caucasian test persons older than 18 years submitted to 30% or 15% xenon for 45 minutes. The study was registered at the European Medicines Agency (EudraCT-number: 2014-000973-38), at ClinicalTrials.gov (NCT number: 02129400) and has been published.[11] For this intervention, Xenon pro Anesthesia from Air

Liquide (Düsseldorf, Germany) and oxygen (Conoxia) from Linde AG (München, Germany) were used. The car bulb containing xenon (M-Tech Basic D1S 4300) was purchased at AutoCouture (Cugy, Switzerland). The airtight gas syringes were purchased from Vici (Baton Rouge, USA); the gas-tight vials (20 mL), from Agilent (BGB Analytic SA, Genève); and EDTA tubes (2.7 mL), from Sarstedt (Nümbrecht, Germany).

Instruments and conditions

The analyses were conducted on a Varian CP-3800 gas chromatograph (Walnut Creek, CA, USA) coupled to a Varian 1200L MS/MS triple quadrupole mass spectrometer (Walnut Creek, CA, USA) operating in electron ionization (EI) mode (70 eV).

The compounds were separated on a series of two columns, a fused silica Restek Rxi®-624Sil MS column (midpolarity Crossbond® silarylene phase) similar to a 6% cyanopropylphenyl/94% dimethylpolysiloxane stationary phase (30 m length × 0.25 mm I.D. × 1.4 µm film thickness) coupled inline to an Agilent J&W (DB-624) with 6% cyanopropylphenyl/94% dimethyl polysiloxane stationary phase (30 m length × 0.32 mm I.D. × 1.8 µm film thickness).

The instrument temperature was 250°C for the transfer line and injector and 160°C for the ion source. Injections were conducted manually with an airtight gas syringe in splitless mode. The initial oven temperature of 40°C was held for 3 minutes, increased by 20°C/minute to 120°C and then kept at this temperature for 2 minutes. The carrier gas was helium with a constant flow of 2.5 mL/minute.

The GC-MS/MS was operated in selected ion monitoring (SIM) mode with the ions *m/z* 84 and 86 for krypton and *m/z* 129 and 131 for xenon for both of the quadrupoles and isolated with ±0.7 amu. The ion source temperature was kept at 160°C. The collision energy was maintained at 20

eV for the transitions *m/z* 84→84, 86→86, 129→129 and 131→131 (there was no fragmentation but a mass cleanup), and the electron multiplier was set to 1 000 V. Argon was used as the collision gas at a pressure of 2.19 mTorr.

Retention times were 4.06 minutes for xenon and 3.95 minutes for krypton.

Data acquisition and MS controls were obtained using the software Varian MS Workstation 6.9.3.

Samples used for validation

*Calibration samples*

Calibration samples were prepared with appropriate volumes of standard xenon and krypton dilutions (1 040 nmol/mL and 52 nmol/mL for xenon; 1 040 nmol/mL for krypton) to obtain six different amounts of xenon injected ranging from 2.08 nmol to 104 nmol (2.08, 5.2, 10.4, 26, 52 and 104 nmol injection volumes). In total, 52 nmol of the internal standard krypton was also added in the gas syringe before injection of each sample. The calibration range was chosen considering the limits of detection for analytical methods of xenon measurement in biological samples, which were already published.[16-18]

*Quality control samples*

Quality control samples were prepared with the appropriate volumes of standard xenon and krypton dilutions (728 nmol/mL and 72.8 nmol/mL for xenon; 1 040 nmol/mL for krypton) to obtain five different injection volumes of xenon ranging from 3.64 to 72.8 nmol (3.64, 7.28,

18.2, 36.4 and 72.8 nmol injection volumes). As for the calibration samples, 52 nmol of krypton was added to the gas syringe before injection of each sample.

*Validation procedure*

The method was validated according the guidelines of the "Société Française des Sciences et Techniques Pharmaceutiques" (SFSTP) commission in 2003-2006.[19] The validation was made with the calibration samples and quality control samples each repeated three times and performed on three non-consecutive days. The parameters considered for the validation were selectivity, calibration curve, linearity, trueness, precision, accuracy, LOD and LOQ to finally obtain an accuracy profile.

The approach was based on the use of a β-expectation interval tolerance of 80%, meaning that the intervals for each experimental point contain an average of 80% of the total values.[20] The tolerance intervals (TI) were defined as $TI = X \pm k \times \sqrt{(S^2_r + S^2_R)}$, where $S^2_r$ is the standard deviation of repeatability and $S^2_R$ is the standard deviation of reproducibility. In the β-expectation interval tolerance approach, $k = t_v \times \sqrt{(1 + [1 / (I \times J \times B^2)])}$, where I is number of series, J is the number of repetitions, and $B^2$ is a coefficient. This coefficient is given as $B^2 = (R + 1) / [J \times (R + 1)]$ with $R = S^2_r / S^2_R$. $t_v$ is Student's coefficient with degrees of freedom $v$ defined as $v = (R + 1)^2 / \{[(R + 1 / J)^2 / (I - 1)] + [(1 - 1/J) / (I \times J)]\}$.

Headspace storage stability study

Bovine blood was placed in six EDTA tubes, and the tube headspaces were fortified with xenon (168 nmol/mL of blood) by direct injection of gas into the headspaces of the tubes through the

septum. All of the tubes were then stored in different places and at different temperatures. One tube was stored at room temperature; one at 4°C; one in a car trunk that was later submitted to a 1 000-km trip under car temperature for two weeks; and three, at -20°C. GC-MS/MS analyses were conducted on four of the samples (room temperature, 4°C, car trunk and -20°C) on the first day and then after two weeks to evaluate the influence of storage temperature and transport on xenon in blood.

GC-MS/MS analyses were conducted on the three samples stored at -20°C to check the influence of freezing/thawing cycles. One sample was thawed and analyzed three times; another, two times; and the last, only once.

Analyses of human biological samples

Seventeen frozen EDTA tubes containing blood from 3 study test persons after inhalation of xenon (30% of xenon mixed with 60% oxygen and 10% air for two volunteers and 15% of xenon mixed with 75% oxygen for the other) for 45 minutes were provided by the Department of Anaesthesiology at the University Hospital RWTH, Aachen, Germany; the blood samples were collected at different sampling times (directly after, 2 h, 4 h, 8 h, 24 h and 48 h after the intervention).[11] To analyze these samples, the EDTA tubes were weighed, and the empty tube weight value was subtracted from the total weight to obtain the blood mass and volume and then the headspace volume. Then, 100 µL of the headspace was sampled and injected in the presence of the internal standard.

## Results and discussion

### Limit of detection (LOD)

The LOD was evaluated by analyzing gas-tight vials containing various decreasing concentrations of xenon. The LOD was considered the lowest analyte concentration that has been evaluated to give a minimal signal-to-noise ratio (S/N) of 3 over at least ten repetitions. The LOD was then calculated at 52 pmol on-column.

### Response function

The response function is described by the relationship between the amount of xenon in the sample and the area ratio of xenon to krypton (analyte to internal standard ratio) within the range of the amount and was defined using calibration samples. Three calibration curves were made on three non-consecutive days ($p=3$) at six levels ($k=6$): 2.08, 5.2, 10.4, 26, 52 and 104 nmol; each was repeated three times ($n=3$). For these calibration curves, a linear relationship ($y= ax+b$) was established between the amount injected and the area ratio of xenon to krypton.

### Linearity

To test the linearity of the method, a linear regression was applied between the back-calculated xenon levels from the quality control samples and the theoretical levels. The quality control samples were measured for five amounts of xenon injected ($k=5$), and each was repeated three times ($n=3$) on three non-consecutive days ($p=3$) (Table 1). The coefficient of linear correlation for the range [2.08–104 nmol] was found to be satisfactory.

For the range of 2.08–104 nmol, both the calibration point and controls were close to the calibration line for the three non-consecutive days, which led to satisfactory correlation coefficients. It should be noted that all of the injections were carried out manually so the results obtained could be considered satisfactory.

Trueness

The trueness or bias is the difference between the estimator's expected value and the true value of the parameter being estimated. The values are shown in Table 1.

According to these values, this method is accurate above 7.28 nmol for the range of 2.08–104 nmol.

Precision: repeatability and intermediate precision

The precision was obtained by conducting multiple analyses of the same sample on three different days and is expressed by the $CV_r$ and $CV_R$. This value was calculated using repeatability (intra-day precision) and intermediate precision (inter-day precision) at each quality control amount of xenon. The repeatability component of the variance was estimated by measuring intra-day variance, and the intermediate precision component of the variance was obtained from the inter-day variance. The values are shown in Table 1.

Accuracy and limit of quantification (LOQ)

The general term ‘accuracy’ is used to describe the closeness of a measurement to the true value and is the sum of systematic error (trueness) and random error (precision). The accuracy profile

of xenon was established according to the proposals of the SFSTP for the harmonization of analytical method validation[19] with a β-expectation tolerance interval set at 80% (Figure 1). From the accuracy profile, the LOQ was defined as 15 nmol on-column for the range of 2.08–104 nmol.

Selectivity

To test the xenon chromatogram selectivity, GC-MS/MS analyses in SCAN mode (50-150 amu) were conducted with xenon in presence of numerous gases: argon (M=39.9 g/mol), nitrous oxide (M=44 g/mol), 3.2 LPG (25% propane, 55% butane and 20% isobutane, M=44.1 and 58.1 g/mol) and a gas (containing $H_2$, $CH_4$, $H_2S$, $CO_2$, $N_2$ and $O_2$) from the abdominal cavity of an altered body.

According to the chromatogram, there were no artifacts or interferences from these gases with xenon.

Another screening in SCAN mode (50–150 amu) was made with xenon, krypton and the following volatile compounds: dichloromethane (M=84.9 g/mol), trichloroethane (M=133.4 g/mol) and trichloroethylene (M=131.4 g/mol). These solvents were chosen due to their similar *m/z* values compared with xenon and krypton, and they were diluted in mineral oil to obtain a concentration of 2 g/L and heated at 80°C for ten minutes.

Then, a screening in SIM mode was performed with the same mixture (xenon, krypton, dichloromethane, trichloroethane and trichloroethylene) with the specific ion transitions of xenon and krypton (84→84, 86→86, 129→129 and 131→131) with a larger time range. The chromatogram is shown in Figure 2.

According to these chromatograms, even if the volatile compounds had similar *m/z* values, they did not interfere with xenon and krypton. With these GC parameters, dichloromethane has a retention time of 7.35 minutes; trichloroethane, of 8.97 minutes; and trichloroethylene, of 9.74 minutes, while the peaks of xenon and krypton had retention times of 4.06 and 3.95 minutes, respectively, so there is no coelution. The chromatogram and mass spectra are specific to xenon.

The methods have been applied to a car bulb containing xenon (M-tech Basic D1S 4300) as an external quality control for xenon detection. The method was practical for the car bulb and showed the 129 and 131 ions of xenon. Nonetheless, due to the unknown volume and pressure in the bulb, it was not possible to determine the amount of xenon contained within.

Storage stability

The influence of storage for 15 days was evaluated with four samples analyzed by GC-MS/MS on day 0 and again after two weeks. Losses of xenon in frozen samples (-20°C) were the weakest (only 2% xenon). Losses of xenon in samples stored in fridge (4°C), at room temperature (close to 22°C) or at environmental temperature (day temperature > 25°C, night temperature > 15°C) experienced greater losses at 61, 77 and 98%, respectively.

However, a more complete study of stability should be carried out on additional samples to confirm these results. Considering the gaseous nature of xenon, frozen storage should be the best storage to prevent xenon loss.

Similarly, the influence of freezing/thawing cycles without tube opening was studied for 15 days. The loss of xenon after three cycles was approximately 50%. Of course, to prevent xenon loss, it is desirable to reduce freeze/thaw cycles to the minimum number possible. The loss of xenon

could also be due to thermal influence on the airtightness of the rubber septa of blood tubes that have been already pierced for the fortification.

Analyses of human biological samples

It should be noted that all samples were from a 2015 study and had therefore already been analyzed, which implies that the tubes were likely opened or penetrated by needles through the septum; thus, there was no longer a guarantee of airtightness. The total storage period was between 6 and 18 months. Therefore, the goal of this application was more focused on the possibility of detecting xenon in human blood under sub-optimal conditions rather than providing precise xenon concentrations. In addition, even though thawing did not strongly impact xenon content, it was still a source of xenon loss (approximately 2% by freezing/thawing) as were the freezing/thawing cycles.

Xenon was detected in 15 samples out of 17. A chromatogram from one of the volunteers subjected to xenon anesthesia is shown in Figure 3.

The results of the analyses of the blood samples are shown in Table 2. As expected, the probes taken directly after chirurgical intervention had the highest xenon concentration, and the maximum was detected in volunteer 2. However, as the conditions of the analyses by the University Hospital RWTH are not entirely known and the tubes were already used, it is difficult to draw conclusions regarding the concentrations. However, these measurements were useful for evaluating the efficiency of detection for xenon.

As a result, the method was shown to be practical for detecting xenon in human blood probe samples up to 48 h after the intervention and after storage under freezing for several months.

As no cut-offs have been set until now, we recommend the use of this method only for detection to diagnose xenon misuse in doping. The method presented herein is one of the most sensitive methods for xenon detection considering an LOD of 52 pmol on-column. Although the concentrations used to induce medical anesthesia are much higher, the calibration range [2.08–104 nmol] is satisfactory for monitoring xenon elimination after xenon exposure.

## Conclusions

In this study, a selective and sensitive method of xenon detection was developed and validated using GC-MS/MS. The method was validated using accuracy profiles and according to the guidelines of the French Society of Pharmaceuticals Sciences and Techniques (SFSTP). The validity of the method was tested using blood from healthy study test persons receiving sub-anesthetic doses of xenon by inhalation. The gas was reliably detected in nearly all the probes even though they were already used. As no cut-offs have been set until now and detection is only recommended to diagnose xenon misuse in doping, the method presented herein is one of the most sensitive for xenon detection considering an LOD of 52 pmol on-column. Because the concentrations used to induce medical anesthesia are much higher, the calibration range [2.08–104 nmol] is satisfactory, and the method provided accurate and reliable estimates over a xenon range of 15 to 104 nmol, which demonstrates the applicability of the method for xenon elimination monitoring after exposure.

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Figure legends

**Fig. 1:** Accuracy profile for the range of 2.08–104 nmol with a β-expectation tolerance interval set at 80% and acceptance limit at ±30%

**Fig. 2:** Screening in SIM mode with krypton, xenon, dichloromethane, trichloroethane, trichloroethylene and the ion transitions 84→84, 86→86, 129→129 and 131→131

**Fig. 3:** Chromatograms coming from volunteer 2 (30% xenon/60% oxygen/10% air for 45 minutes) immediately after inhalation of xenon

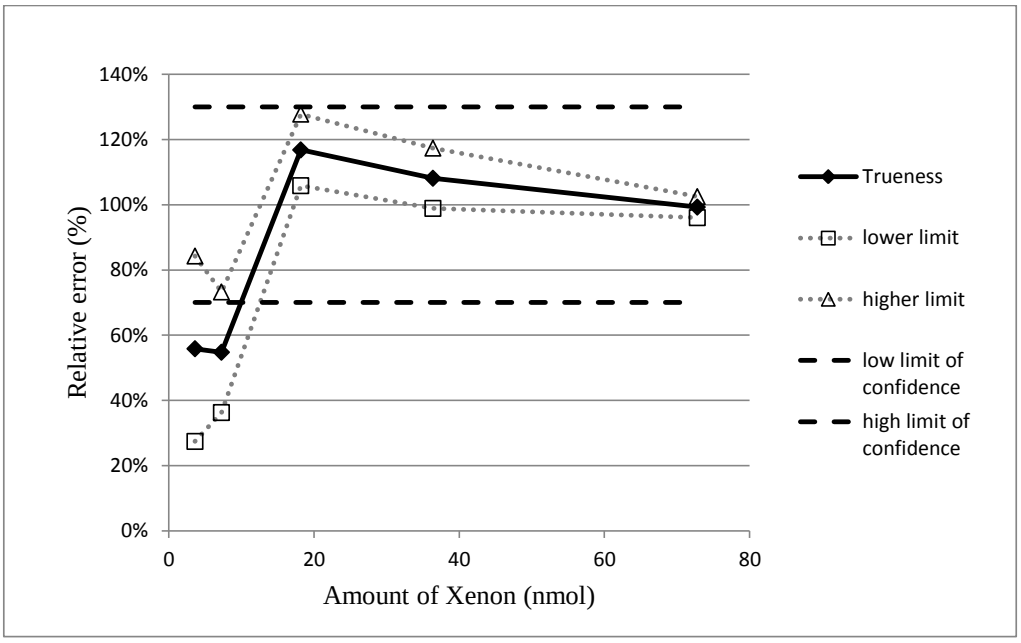


Figure 1: Accuracy profile for the range of 2.08–104 nmol with a β-expectation tolerance interval set at 80% and acceptance limit at ±30%

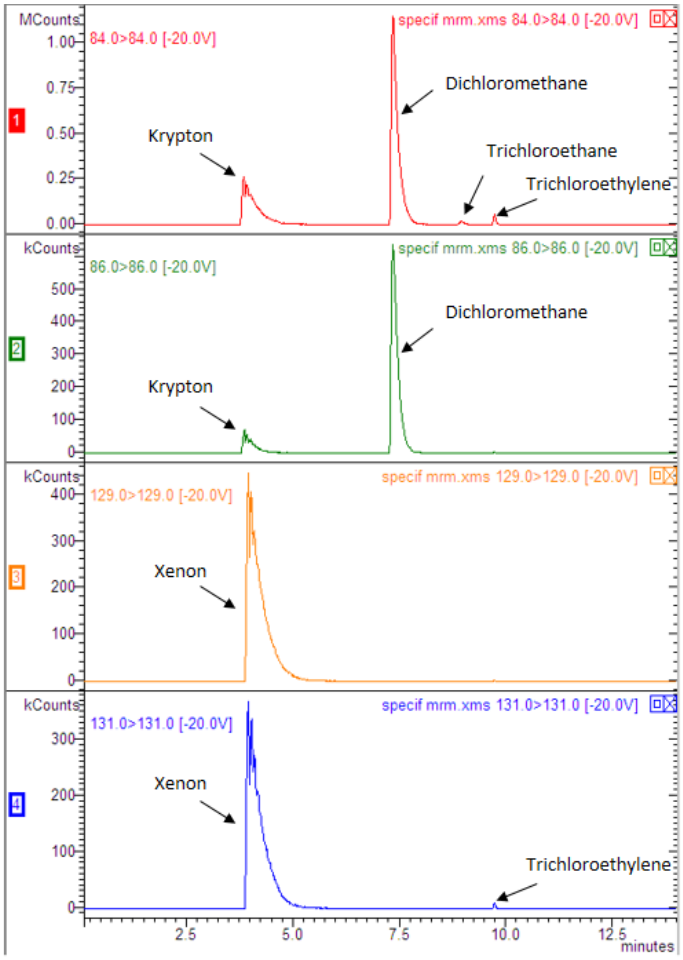


Figure 2: Screening in SIM mode with krypton, xenon, dichloromethane, trichloroethane, and trichloroethylene and with the ion transitions 84→84, 86→86, 129→129 and 131→131

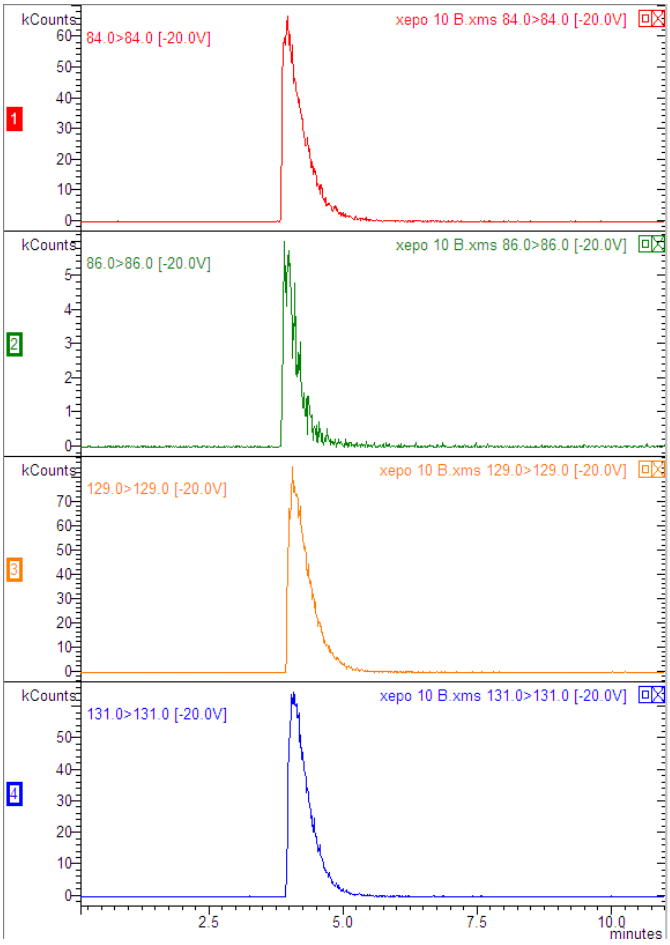


Figure 3: Chromatograms coming from volunteer 2 (30% xenon/60% oxygen/10% air for 45 minutes) immediately after inhalation of xenon

Table 1. Results from the method validation

**Range [2.08–104 nmol]**

| | Day 1 | Day 2 | Day 3 |
|---|---|---|---|
| Slope | 0.262 | 0.045 | 0.041 |
| Intercept | - 0.456 | 0.089 | 0.058 |
| $R^2$ | 0.996 | 0.992 | 0.991 |

**Range [2.08–104 nmol]**

| Target value | 3.64 | 7.28 | 18.2 | 36.4 | 72.4 |
|---|---|---|---|---|---|
| Level average | 2.033 | 3.987 | 21.26 | 39.37 | 72.28 |
| Bias | - 44% | - 45% | 17% | 8% | - 1% |
| Lower limit | 0.998 | 2.641 | 19.27 | 36.00 | 69.93 |
| Upper limit | 3.068 | 5.334 | 23.26 | 42.73 | 74.64 |
| CVr | 23.77% | 15.77% | 6.34% | 5.43% | 2.28% |
| CVR | 23.70% | 16.15% | 6.35% | 5.19% | 2.11% |

Table 2. Results of the analyses of blood samples from patients after exposure to xenon anesthesia (LOD: 52 pmol, LOQ: 15 nmol)

| **(nmol/mL of blood)** | **Directly after** | **2 h** | **4 h** | **8 h** | **24 h** | **48 h** |
|---|---|---|---|---|---|---|
| **Volunteer 1** | 19 | No sample | < LOQ | < LOQ | < LOQ | < LOQ |
| **Volunteer 2** | 130 | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ |
| **Volunteer 3** | 22 | < LOQ | < LOQ | < LOQ | Not detected | Not detected |

**Figure 1**

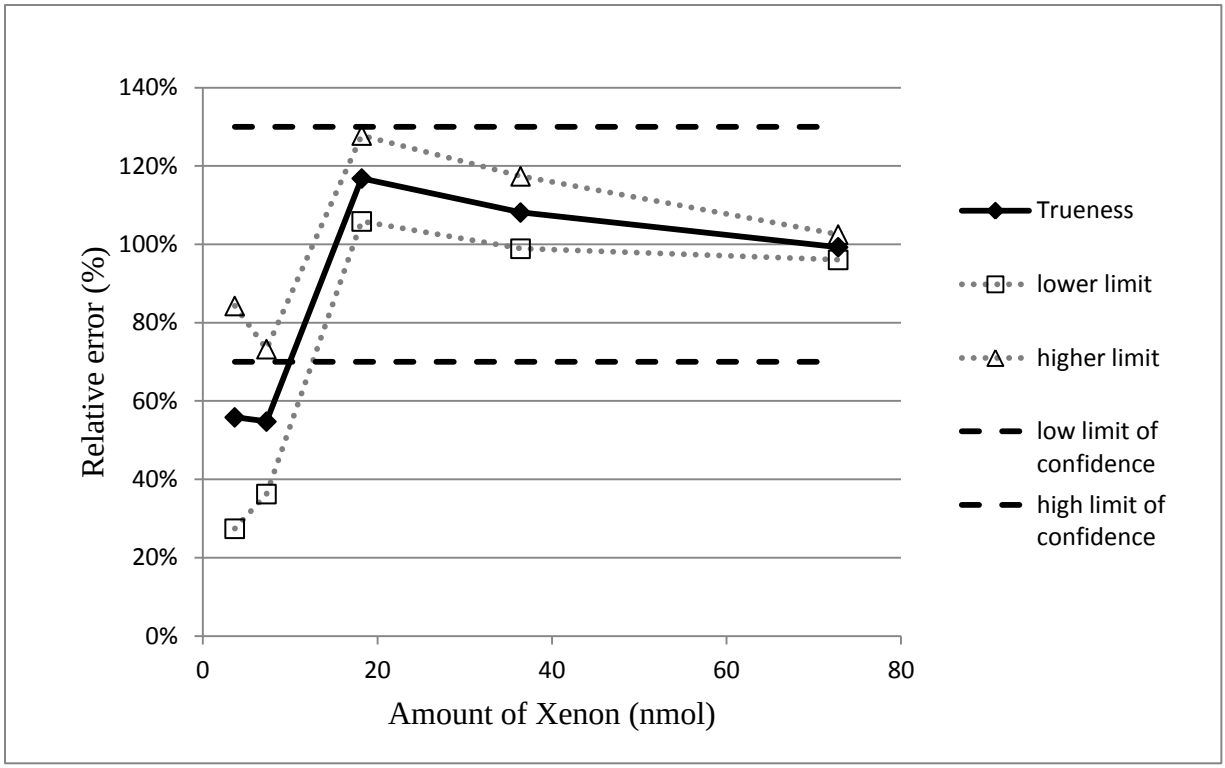


Figure 1: Accuracy profile for the range 2.08-104 nmol with a β-expectation tolerance interval set at 80% and acceptance limit at ±30%

**Figure 2**

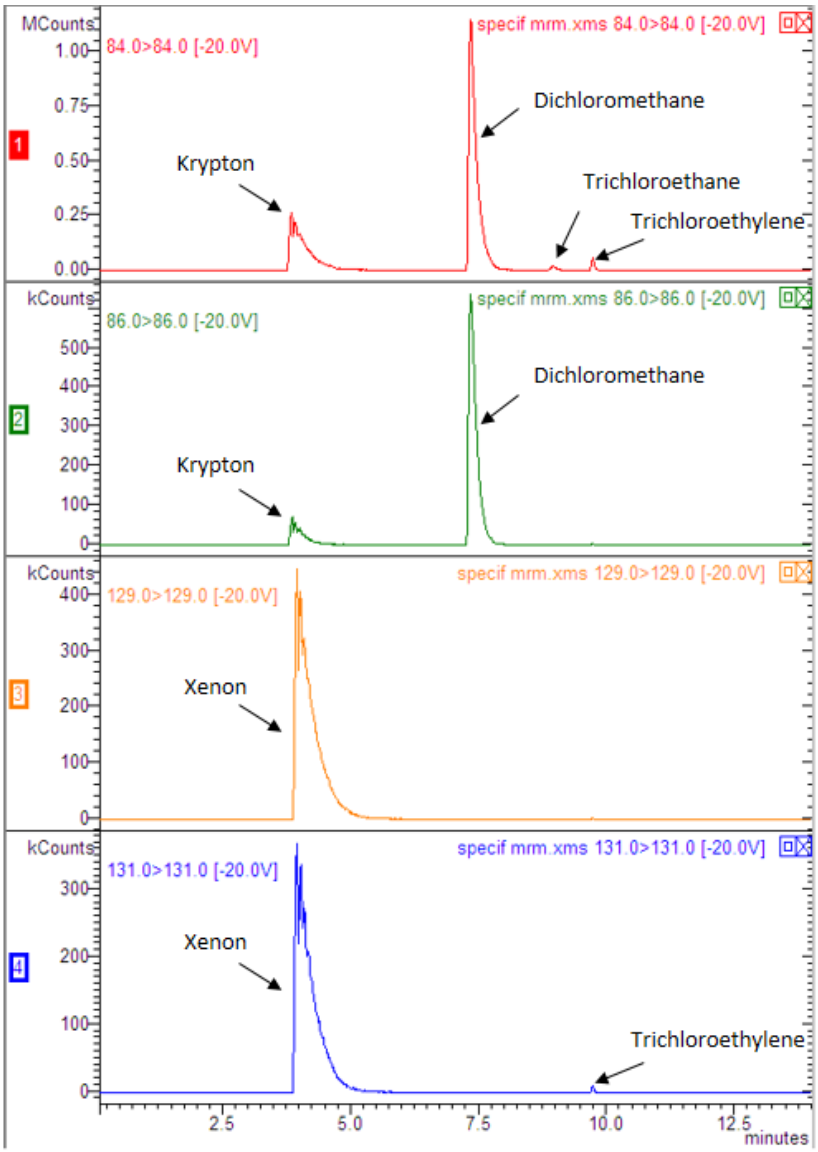


Figure 2: Screening in SIM mode with krypton, xenon, dichloromethane, trichloroethane, trichloroethylene and the ion transitions 84→84, 86→86, 129→129 and 131→131

**Figure 3**

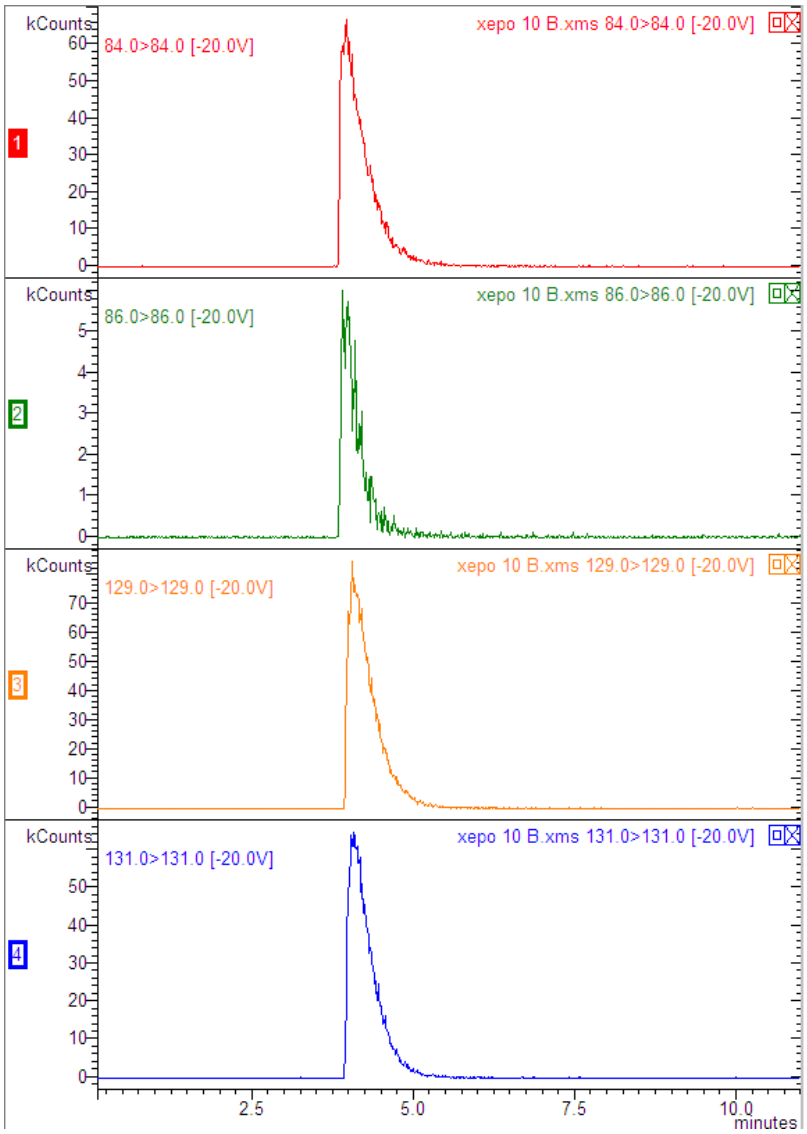


Figure 3: Chromatograms coming from volunteer 2 (30% xenon/60% oxygen/10% air for 45 minutes) immediateley after inhalation of xenon

**Table 1**

Table 1. Results from the method validation

| | **Range [2.08 – 104 nmol]** | | |
|---|---|---|---|
| | Day 1 | Day 2 | Day 3 |
| Slope | 0.262 | 0.045 | 0.041 |
| Intercept | - 0.456 | 0.089 | 0.058 |
| $R^2$ | 0.996 | 0.992 | 0.991 |

| | **Range [2.08 – 104 nmol]** | | | | |
|---|---|---|---|---|---|
| Target value | 3.64 | 7.28 | 18.2 | 36.4 | 72.4 |
| Level average | 2.033 | 3.987 | 21.26 | 39.37 | 72.28 |
| Bias | - 44% | - 45% | 17% | 8% | - 1% |
| Lower limit | 0.998 | 2.641 | 19.27 | 36.00 | 69.93 |
| Upper limit | 3.068 | 5.334 | 23.26 | 42.73 | 74.64 |
| CVr | 23.77% | 15.77% | 6.34% | 5.43% | 2.28% |
| CVR | 23.70% | 16.15% | 6.35% | 5.19% | 2.11% |

**Table 2**

Table 2. Results of the analyses of blood samples from patients after exposure to xenon anaesthesia (LOD : 52 pmol, LOQ : 15 nmol)

| **(nmol/mL of blood)** | **Directly after** | **2h** | **4h** | **8h** | **24h** | **48h** |
|---|---|---|---|---|---|---|
| **Volunteer 1** | 19 | No sample | < LOQ | < LOQ | < LOQ | < LOQ |
| **Volunteer 2** | 130 | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ |
| **Volunteer 3** | 22 | < LOQ | < LOQ | < LOQ | Not detected | Not detected |