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Short Communication

A method for the determination of the residual chloroform in defatted cancellous bone transplants

Ulrich Kalus[1], Helga Müller[2], Hans Baudisch[2], Hans-Jürgen Birkhahn[2], Rüdiger von Versen[3], Arne Hansen[1] and Axel Pruss[1,*]
*[1]Institute for Transfusion Medicine (Tissue Bank), University Hospital Charité, Berlin, Germany; [2]Institute for Toxikology (BBGes), Berlin, Germany; [3]DIZG - German Institute for Cell and Tissue Replacement, Berlin, Germany; *Author for correspondence (e-mail: axel.pruss@charite.de; phone: 49-30-450525161; fax: 49-30-450525976)*

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Abstract

The removal of fat from cancellous bone tissue promotes the clinical healing of the transplant and improves the penetration of chemical sterilisation media into the tissue. Treatment using chloroform/methanol (2:1, 2 h) is frequently used as a defatting procedure. Eight rinses with methanol followed by two rinses with aqua ad iniectabilia (20 min each, with ultrasonic effect) ensure depletion in the level of chloroform from defatted cancellous bone to a concentration below 25 ppm (limit value). For the necessary routine quality checks on the production process, a gas chromatography method has been developed that determines the level of chloroform in cancellous bone, for which the detection limit is 0.003 ppm.

Introduction

Cancellous bone tissue contains a high level of fat. This fat is undesirable in terms of both the clinical bone healing (Aspenberg and Thoren 1990; Thoren and Aspenberg 1995; Thoren et al. 1995) and the use of chemical sterilisation procedures. While in this connection, the effect of these fatty components is relatively insignificant during the radiation treatment and thermal disinfection of transplants – at least in terms of the effectiveness of the procedure – (Pruss et al. 2002, 2003a, b), they form a penetration barrier that prevents chemical sterilisation solutions from penetrating into the cancellous tissue (von Versen et al. 1993).

In the tissue bank at the Charité, a chemical cold sterilisation procedure based on peracetic acid/ethanol is used (von Versen and Starcke 1989; von Versen et al. 1993; Pruss et al. 2003c). For this reason, cancellous transplants in particular must be defatted prior to sterilisation. The defatting method consists of the application of a chloroform/methanol mixture at a ratio of 2:1 over 2 h. To remove the chloroform, the transplants then undergo eight methanol rinses of 20 min each in an ultrasonic bath. Aqua ad iniectabilia rinses are then applied twice to remove the methanol. In other facilities pressure treatment with warm water is also used instead of the chloroform/methanol defatting procedure.

Different authors have studied the toxic effect of chloroform (trichlormethane, $CHCl_3$) in relation to the duration of exposure. In animal experiments, damage to the liver and kidneys occurs following long-term exposure (>14 days) to a chloroform dose of 25 mg/kg KG/day and above

(Agency for Toxic Substances and Disease Registry - ATSDR, 1989). Other authors indicate kidney and liver tumours in rats from doses of 90 mg/kg KG and above over a period of 78 weeks with exposure at 5 days/week (NCI 1976; Reubner 1979). Jorgenson et al. (1985) already describe kidney and liver tumours in the mouse model for doses of 19 mg/kg KG and above. However, this dose was applied daily over a period of 104 weeks. Consistent mutagenic and reproduction-toxic effects were only detected following higher doses over a long period of time (Nagano et al. 1998; Greim 1999).

In summary, the ATSDR has concluded that a minimal risk to health exists following long-term exposure to doses of 0.03 mg/kg KG and above. For a person weighing 70 kg, this would mean a daily intake of 2.1 mg of chloroform over a period of at least 14 days. A risk of cancer of 1:100,000 from a chloroform dose of 0.01 mg/kg KG and above over a long time period has been established (WHO 1994). For a person weighing 70 kg, this would correspond to a daily intake of 0.63 mg of chloroform.

No standard levels have been defined for the permitted residual chloroform concentration in the cancellous transplant. In the Charité tissue bank, a maximum residual chloroform level of 25 ng/mg (ppm) is permitted in cancellous transplants. Hassauer (1993) defines the average chloroform intake of a person living in central Europe via all polluting media (foodstuffs, drinking water, room air, external air) as being approximately 1.5 μg/kg KG/d. For a person weighing 70 kg, this means an intake of approximately 0.1 mg of chloroform per day. For defatted transplants, the limit value of 25 ppm corresponds to a maximum level of 1.5 mg of chloroform per transplant, depending on weight. This is comparable to the common level of chloroform pollution from the environment over approximately 14 days.

The aim of the studies was to develop a method based on gas chromatography that can consistently determine a residual level of chloroform of <25 ppm in defatted cancellous transplants. A target detection limit of <1 ppm was specified to ensure the quality of the production process. A test was also planned to ascertain whether the routine methanol rinses following defatting are capable of guaranteeing that residual chloroform levels are below 25 ppm, and whether the effect of the ultrasonic during the rinses is beneficial.

Material and methods

Methods to determine the chloroform content

Reagents

Chloroform p.a. (LGC Promochem), methanol p.a. (Merck), 2-brom-1-chloropropane p.a. (Merck)

Standard solutions

Stock solution. To a 10 ml volumetric flask filled 2/3 with methanol, approximately 100 μl of precisely weighed chloroform is mixed and made up to 10 ml with methanol. The same procedure is carried out with the inner standard 2-brom-1-chloropropane. Both solutions contain approximately 15 μg/μl (15,000 ng/μl) of each substance and can be preserved for at least 3 months when stored in a refrigerator.

Standards I and II. The chloroform solution (15,000 ng/μl) is diluted with methanol (1:100). The standard I contains 150 ng/μl of chloroform. From this standard, two further dilutions are carried out with methanol, 1:10 (standard IA) with the chloroform concentration 15 ng/μl, and 1:20 (standard IB) with the chloroform concentration 7.5 ng/μl. The parent solution of the internal standard is diluted with methanol (1:100). This standard II contains 150 ng/μl 2-brom-1-chloropropane. All standards can be preserved for at least 3 months when stored in a refrigerator.

Working standard (control solution, internal quality control). About 50 μl of standard IA is pipetted into a 25 ml volumetric flask with 20 ml of bidistilled water, and 50 μl of standard IB is pipetted into a second flask, also with 20 ml of bidistilled water. Both volumetric flasks are then each pipetted with 50 μl of standard II and filled up to 25 ml. After they have been filled up: working standard I contains 30 ng/ml of chloroform; working standard II contains 15 ng/ml of chloroform; both working standards I and II contain 300 ng/ml of 2-brom-1-chloropropane. The working standards are carried out at the beginning

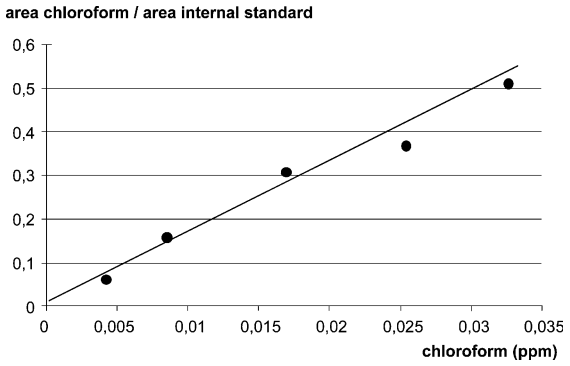

Figure 1. Calibration curve for chloroform quantitation.

and the end of every measuring series (8–12 samples).

The measurement procedure

Calibration curve. For quantitation, a 5-point curve of chloroform (trichloromethane) in Aqua bidest. was constructed (0.004; 0.008; 0.016; 0.024; 0.032 ppm, see Figure 1). Each calibration was linear in the range tested. The limits of quantitation (LOC) were 0.01 ppm. The limit of detection (LOD) was < 0.003. The correlation coefficient was 0.992. The calibration range was adapted to the anticipated low chloroform concentrations following the rinses. As a statistical evaluation program for calibration, the software B.E.N. ('Berechnung/Erfassung/Nachweisgrenze' – calculation/recording/detection limit), version 2.01 (Heidelberg) was used. The program conforms to the DIN 32645 German industry standards.

Measuring methods. Weigh in into a 25 ml volumetric flask four times for every 50–60 mg of the tissue to be tested. Add 20 ml of water and 50 μl of standard II (as internal standard: 2-brom-1-chloropropane) into all flasks. Close the flasks and expose to the ultrasonic 4×15 min, allow to cool and fill up to 25 ml. Pipette 1 ml from each sample solution and the working standards I and II into a head-space glass, and close. Incubate all vessels for 15 min at 80 °C in the head-space sampler. Analyse in the gas chromatograph. The weight of ng chloroform/mg of tissue is given in ppm. The result is the average value of the four measurements.

Apparatus. Gas chromatograph GC-14A with ECD and head-space autosampler AOC-5000 (Shimadzu); GC-column: Permabond OV-624-DF 1.80 μm, 25 m $\times$ 0.32 mm (Macherey–Nagel); Sonorex ultrasonic bath, Super RK 255H (Bandelin); 10 ml head-space glass (Macherey–Nagel); Stainless steel closing cap with PTFE/SILICON sealing (Merck).

Measuring conditions. Start temperature 40 °C, then heat with 3 °C/min up to 100 °C and maintain for 2 min, final temperature 85 °C, detector temperature 150 °C, injector temperature 150 °C. Retention times: 4.2 min (chloroform) and 9.6 min (2-brom-1-chloropropane).

Determining the effectiveness of the methanol rinses

Preparation and defatting of the test samples (bone spongiosa)

Potential bone tissue donors underwent clinical examination for a variety of infectious diseases: virus hepatitis, tuberculosis, syphilis, septicaemia, systemic viral disease, and mycoses, demonstrable at time of death. Positive donors, as well as those with malignoma, those treated previously with human growth factor preparations, dura mater transplant recipients, and individuals fulfilling other exclusion criteria by the European Association of Tissue Banks were excluded. Every bone tissue donor was tested for hepatitis B virus surface antigen (HBsAg) and antibodies against HIV-1/2, HCV, and *Treponema pallidum* ante or post mortem. Only individuals negative for these markers were considered as donors.

Spongiosa tissue was collected under aseptic conditions from the vertebral column, and the epiphyses of femur or tibia, respectively. Fat and connective tissue was removed carefully under aseptic conditions, using scalpel and surgical tweezers. The spongiosa specimen (chips, 1–3 mm) were cut on a belt saw (FK 22, Bizerba). They were rinsed under high pressure to completely remove blood from the bone tissue using sterile water at 37 °C for 30 min. Any remaining fat was removed by placing the tissue into a mixture of chloroform (extra pure, 99.4%) and methanol (for analysis, 99.8%) (v/v: 2/1) under constant agitation (laboratory shaker THYS 2, MLW) over a period of 2 h by changing the delipidating solution every 30 min. To completely remove any residual chloroform, tissues were

sonicated eight times with methanol in a ultrasonic bath (Sonorex RK 510 H, Bandelin electronic) for 20 min. Methanol was removed by flushing the tissues twice with Aqua ad iniectabilia (Braun) also in a ultrasonic bath.

The residual chloroform level was measured in human spongiosa. To do this, 4×9 ccm of spongiosa chips underwent the defatting procedure described above, 3×3 ccm was removed from each chip after the 3rd, 5th, 7th and 10th rinsing process, and the chloroform was measured in the gas chromatograph. The average value of three measurements was given as the result (statistic evaluation using Excel, Windows 97, Microsoft). The same experiment was repeated without ultrasonic.

Results

After three methanol rinses, the values were still very high (398–400 ppm) both with and without the ultrasonic bath. After the 5th methanol rinse, the ultrasonic begins to have a clear effect (without ultrasonic: 366 ppm, with ultrasonic: 32 ppm). After the 7th rinsing process in the ultrasonic series, levels were already lower than the limit value of 25 ppm specified in the testing instructions. After the 10th rinse, levels were lower than the limit value for both series. In the ultrasonic series, the chloroform concentration lay below the determination limit (see Table 1).

The calibration curve gives precision and linearity within the measuring interval. The linearity of the calibration was confirmed up to 400 ppm. The variation coefficient of the triple determination lay between 5 and 25%. This is acceptable due to the non-homogeneity of the material.

Errors caused by other components (e.g. 1,1,1,-trichlorethane) are not possible, since they are recorded with a different retention time, i.e. they do not interfere with the measuring signal. Non-halogenated hydrocarbons are not recorded by the ECD (electron capture detector).

Discussion

Using the recently developed gas chromatograph method to determine the level of chloroform in human spongiosa, residual concentrations from < 25 ppm to the lowest concentrations can be recorded consistently. This also ensures in a methodical way that the recommended, clinically acceptable limit value for residual chloroform levels in human bone spongiosa that has been defatted with chloroform/methanol is maintained.

When a methanol rinse is applied eight times, and an aqua ad iniectabilia rinse is applied twice in an ultrasonic bath for 20 min each, the removal of chloroform from defatted cancellous transplants to a level of below 25 ppm is ensured. The use of an ultrasonic bath is preferable to simply accelerating the release of chloroform from the tissue through rinsing, and is therefore recommended.

In the approximately 100 measurements that have been carried out since as a part of the routine quality checks at the Charité tissue bank on defatted bone and soft tissue that has been rinsed with methanol or aqua ad iniectabilia, no cases arose where a chloroform concentration above 25 ppm was detected. This meets the requirements regarding validation of the production process, as specified in the standards of the Draft Directive 319 of the Council of the European Union (2003) and in the European Association of Tissue Banks (EATB 2003).

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Table 1. Chloroform concentration (ppm) after rinsing with and without ultrasonic.

Number of rinsing	Chloroform[a] (ppm) (rinsing with ultrasonic)	Chloroform[a] (ppm) (rinsing without ultrasonic)
3	400 (SD 44.28, VK 11.07%)	398 (SD 34.95, VK 8.78%)
5	32 (SD 5.29, VK 16.53%)	366 (SD 22.34, VK 6.10%)
7	21 (SD 3.61, VK 17.19%)	52 (SD 8.18, VK 15.73%)
10	< 0.03 (SD 0, VK 0)	15 (SD 3.61, VK 24.06%)

[a] average from 3 measurements

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