Determination of Regenerated Sarin (GB) in Minipig and Human Blood by Gas Chromatography-Chemical Ionization Mass Spectrometry Using Isotope Dilution and Large Volume Injection

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Introduction

Chemical warfare nerve agent biomarker methods:

- **Cholinesterase activity,**
- **Nerve agent hydrolysis products,**
- **Fluoride ion regenerated alkyl methylphosphonofluoridate (G-agents)**
  - **human plasma,**
  - **red blood cells,**
  - **tissue**

New analytical method:

- **ammonia CI,**
- **a large volume injector (LVI), and**
- **stable isotope ($^{2}H_{6}$-GB) internal standard.**

Objectives

- Analyze ethyl acetate extracts of GB exposed samples using GC-MS with positive ammonia chemical ionization and stable isotope standards.
- Explore performance characteristics including:
  - Column selection
  - optimizing LVI and oven parameters,
  - reportable range,
  - accuracy and precision,
  - detection limits.
- Spike human and animal whole blood at a series of GB levels
  - Determine RBC/Plasma ratio *in vitro*
  - Storage considerations
  - Compare RBC/Plasma ratio to minipig exposure data

Experimental Methods: Materials

- **Sarin (GB)** : CASARM grade prepared and analyzed at ECBC and diluted with hexane.
- **Hexadeuterated Sarin ($(^2H_6)$ isopropyl methylphosphonofluoridate ($^2H_6$GB))**: Synthesized at ECBC
- **C_{18} SPE cartridges:** 200mg (Waters Associates, Millipore Corp., Milford, MA)
- **Acetate buffer (pH=3.5)**
- **Potassium fluoride:** 6 M
- **Chemicals**: All other chemicals were procured commercially at ACS reagent grade or higher.

Minipig Blood Sample Collection

Inhalation Exposure Samples: Whole blood from GB exposed minipigs was collected (with EDTA) via external jugular catheter allowing serial blood sample collection before, during, and after inhalation exposure.

Samples were centrifuged at 4400 rpm for 5 min.The resulting red blood cell pack and serum/plasma samples were analyzed for regenerated agent by the addition of acetate buffer and fluoride ion.

Blood Sample Process Flowchart

RBC + Plasma (w/ Protein bound Isopropyl Methylphosphonyl groups)

4,400 RPM

Acetate Buffer (pH 3.5) + Fluoride (6 M KF) + **$^2H_6$-GB (1000 pg)**

Whole Blood/EDTA
[spiked with agent in hexane, mixed for 10 min]

Free GB/$^2H_6$-GB + in aqueous matrix

C18 SPE cleanup, elute w/ ethyl acetate

Free GB/$^2H_6$-GB in ethyl acetate

Analysis by LVI GC-MS

Amount of GB (pg) in sample from Peak Area Ratio ($GB_{area}/^2H_6\text{-}GB_{area}$)

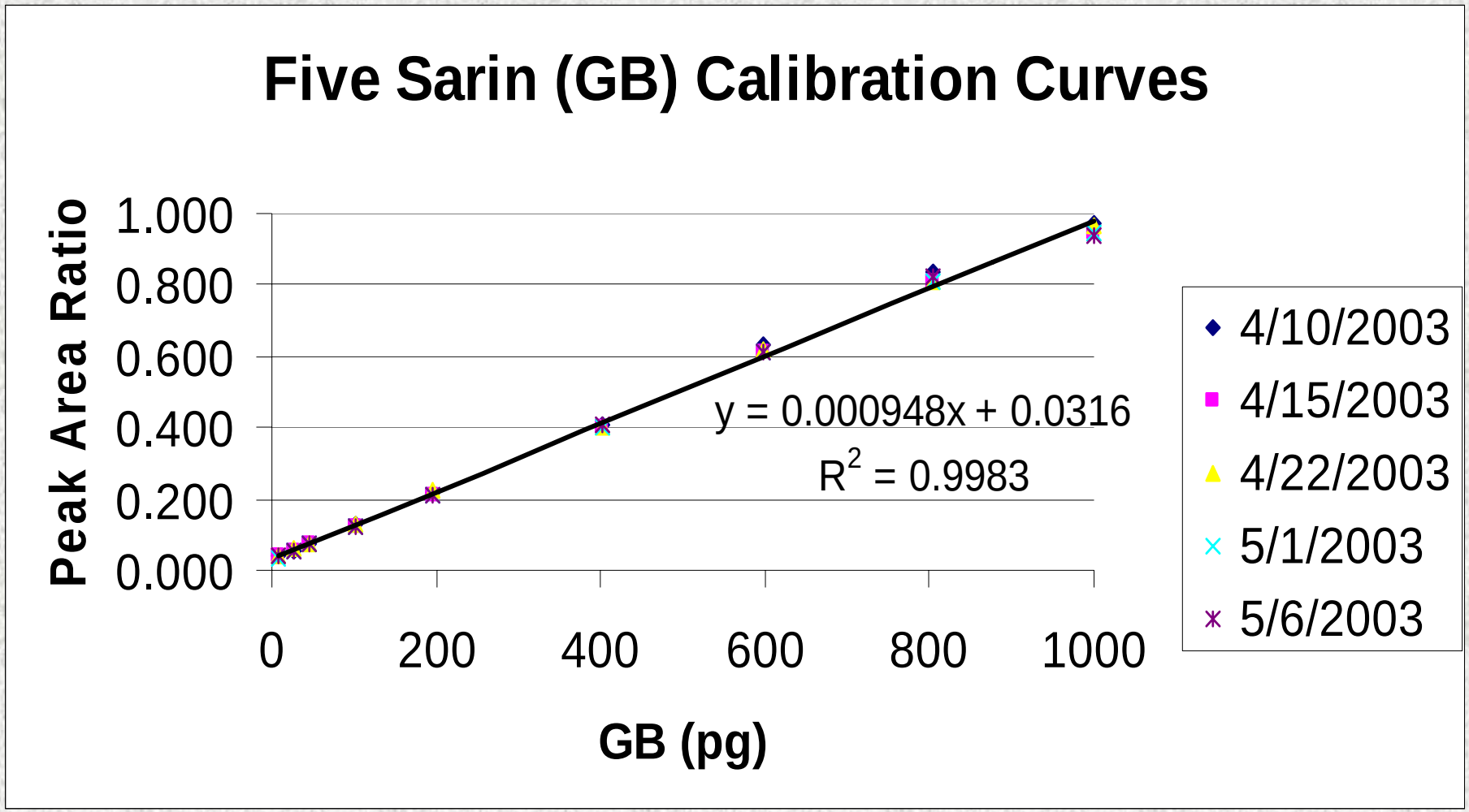
Experimental Methods: Analysis

- **GC** Model 6890 (Agilent Technologies, Wilmington, DE)
- **LVI Sample Introduction:** Agilent PTV: Inject 50 uL of extract, initial temp -20°C, initial time 5.1 min, final temp 225°C, rate 720°C/min, vent time 5.00 min, vent flow 300 mL/min, purge flow 50 mL/min, purge time 8.7 min.
- **GC Columns:**
  - 5%-Diphenyl 95%-Dimethylpolysiloxane: 30 m x 0.32 mm x 1 µm film (HP-5ms, Agilent Technologies, Wilmington, DE)
  - 14%-Cyanopropylphenyl 86%-Dimethylpolysiloxane: 30 m x 0.32 mm x 1 µm film(Rtx-1701, Restek Inc., Bellefonte, PA)
- **GC Oven program:** Carrier He @ 3 mL/min (63 cm/sec),Temp Program: -20°C(9.3 min) to 50°C @ 40°C/min to 64°C @ 2°C/min to 275°C(2 min) @ 50°C/min.
- **Detection:** MSD (Model 5973 MSD, Agilent Technologies, Wilmington, DE) SIM mode, Source & Quad Temp 150°C
  - GB ions: Target-158.1 ($[M+18]^+$) & Qualifier 175.2 ($[M+35]^+$), Retention time 17.67 min
  - Internal standard ($^2H_6$-GB):Target-164.1 ($[M+18]^+$) & Qualifier 181.2 ($[M+35]^+$), Retention time 17.62 min

Results and Discussion

- Positive ion NH_3 chemical ionization (CI) was chosen over CI using negative ion NH_3, positive/negative ion CH_4, or positive/negative ion isobutane.
  - NH_3 afforded better sensitivity then CH_4 or isobutane
  - Positive ion mode less sensitive to source conditions
- Ratio of $[M+18]^+/[M+35]^+$ was used as tuning benchmark to set NH_3 pressure
- LVI flow rates and injector port purge times are critical parameters to optimize for each solvent and target analyte.
- Detection limit was in the high femtogram range.
- Calibration Curve Range 10-1000 pg on column
- Figure 1 demonstrates reproducibility of calibration curves over a month

Figure 1. Calibration Curves



Results and Discussion

- Peak-to-peak signal/noise at the lowest standard (10 pg) was over 150.
- Column preference: The 14%-Cyanopropylphenyl 86%-Dimethylpolysiloxane (RTX-1701) column (B) yielded superior resolution and retention time consistency as compared to the 5%-Diphenyl 95%-Dimethyl- polysiloxane (HP-5MS) column (A) as seen in Figure 2.
- Figure 3 is a representative plot of the d_6-GB spiked extract of blood from a minipig exposure to GB by whole body inhalation.

Figure 2. Column Comparison

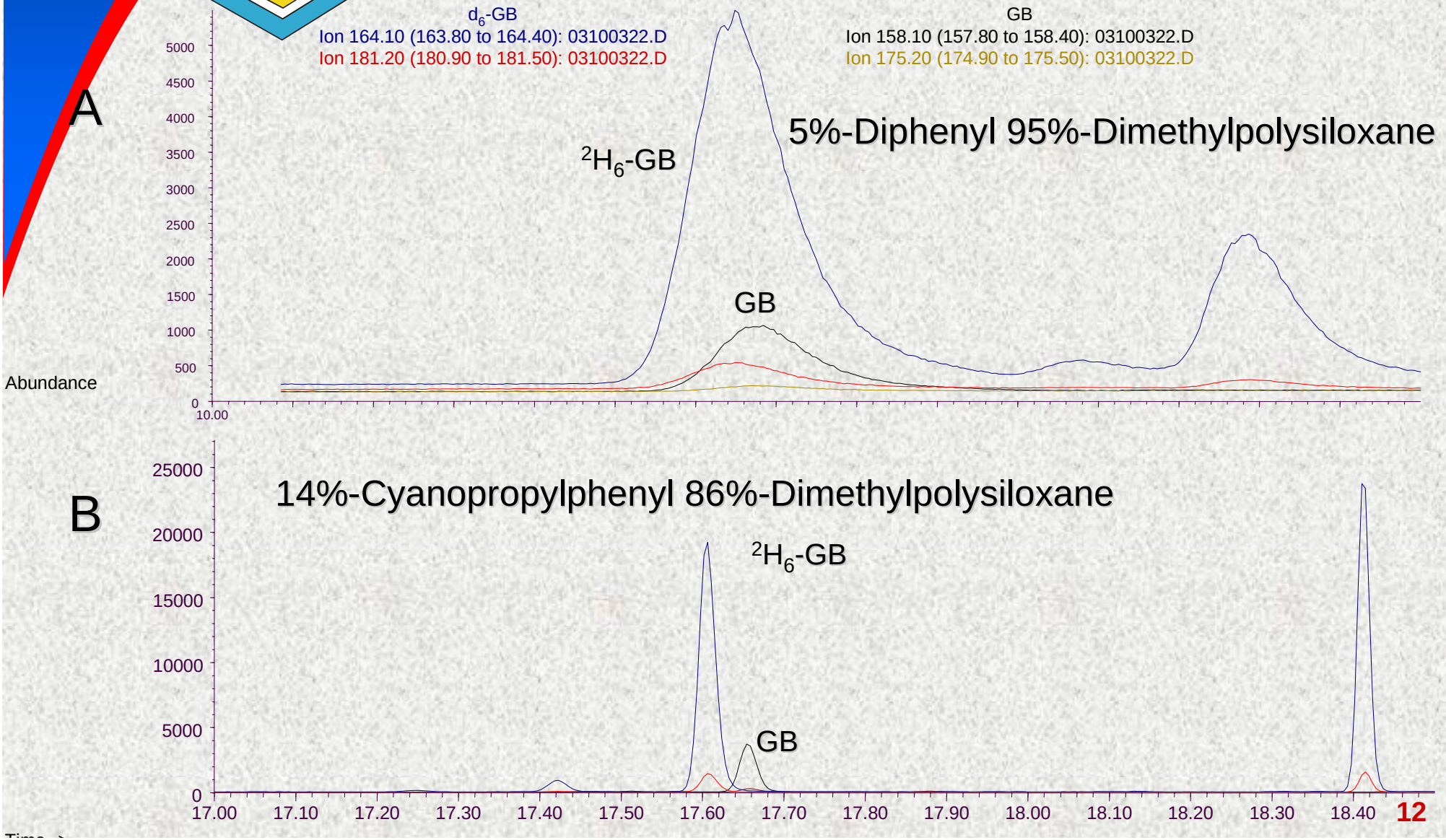
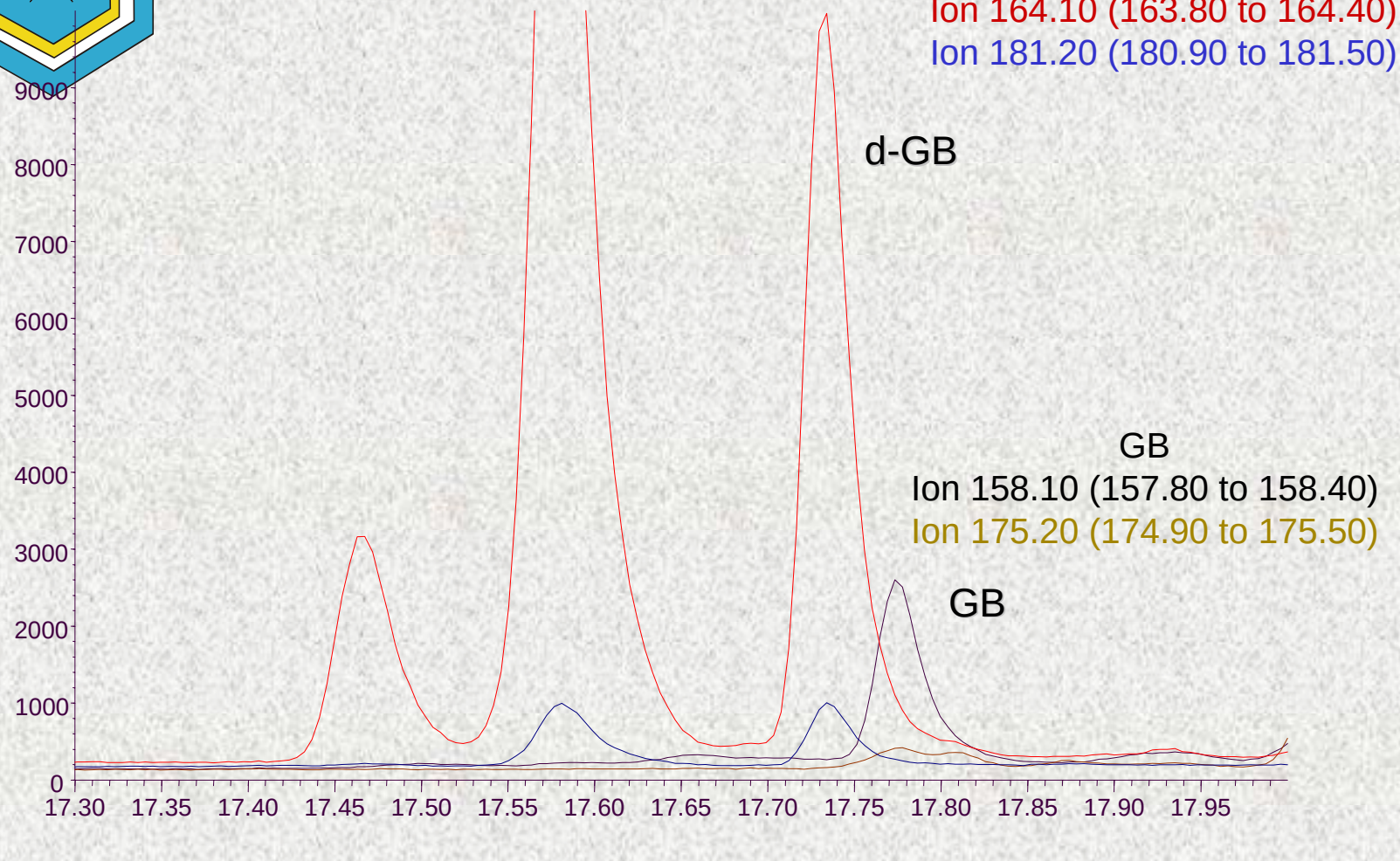


Figure 3. Extract from Exposed Minipig (216 pg GB)



Results and Discussion

- Figure 4 represents a comparison of methods analyzing regenerated GB by either Isotope Dilution GC-MS or External Std GC-FPD:
  - External std method doesn't account for losses that occur during or after sample preparation
  - Deuterated GB isn't resolved well enough even by the Rtx 1701 column to be used as an internal standard for GC-FPD
- Figure 5 demonstrates the recovery of GB from spiked minipig blood for both the HP-5MS and the Rtx-1701 columns for three different levels.
  - Rtx-1701 yielded better precision and accuracy overall.
  - Retention times for GB and d_6-GB varied significantly using the HP-5MS.

Figure 4. Isotope Dilution vs Instrumental Internal Std

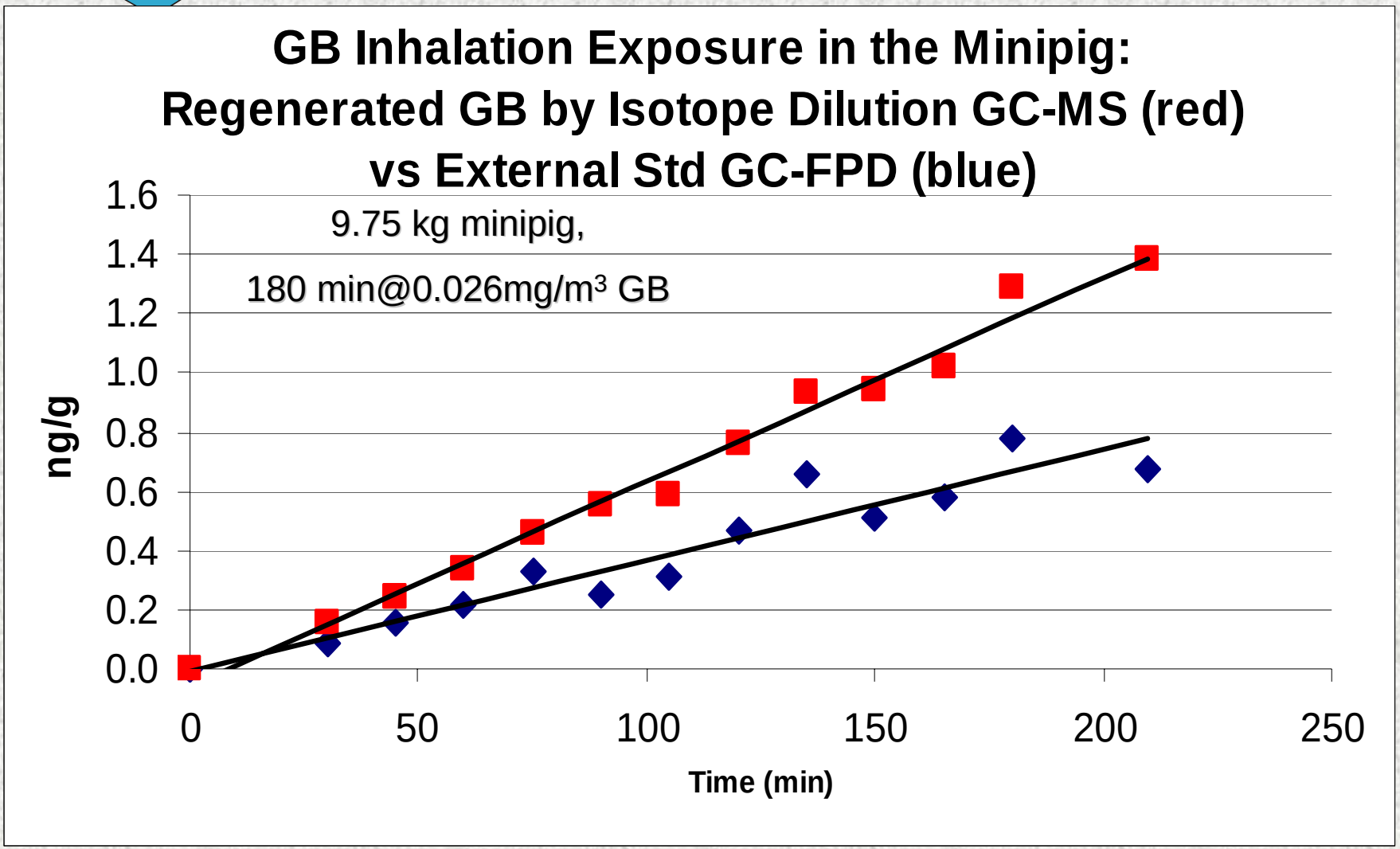
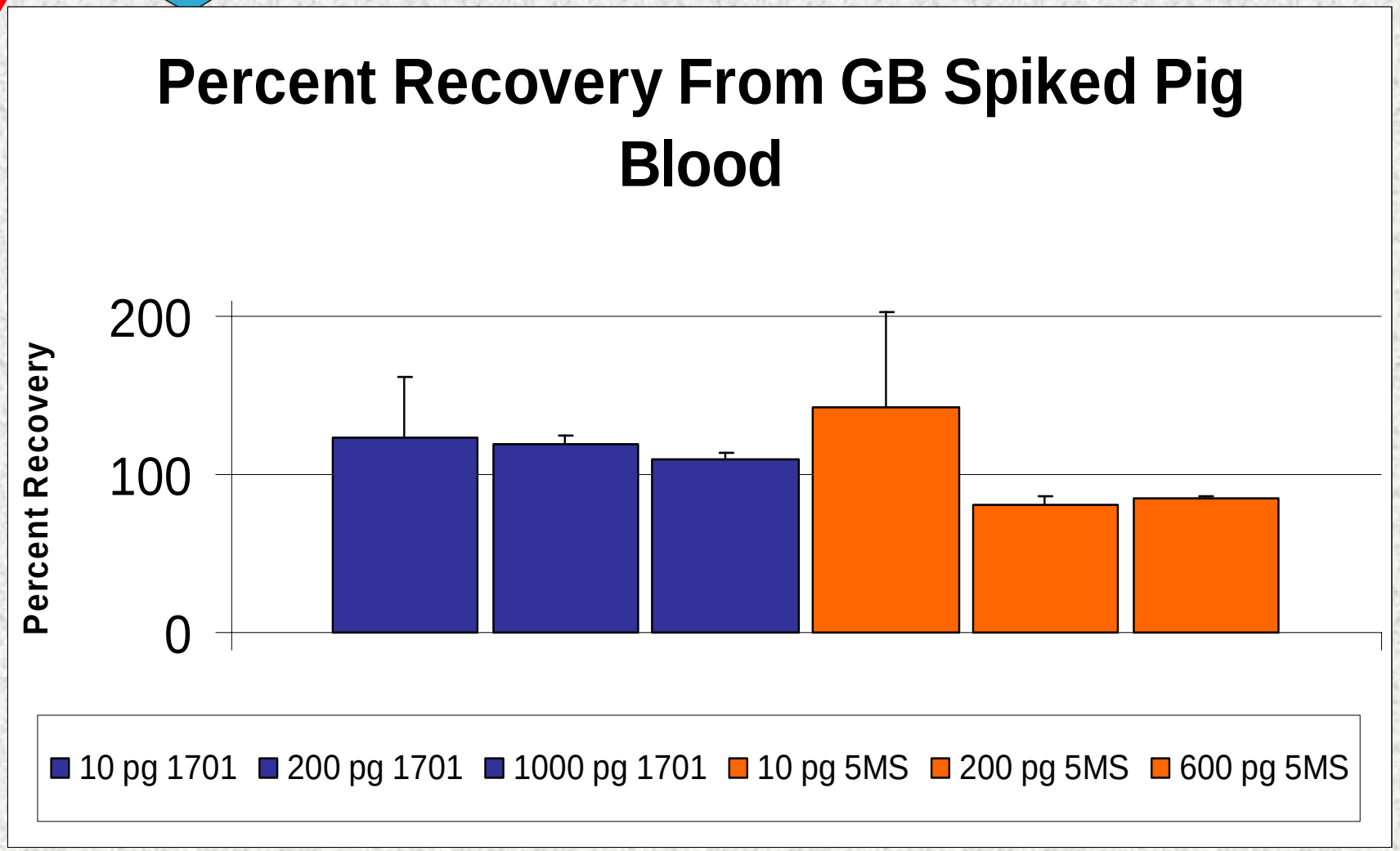


Figure 5. Recovery of GB from Spiked Minipig Blood



Results and Discussion

- Figure 6 shows the recovery of R-GB from human whole blood spiked with 2 ng GB.
- Figure 7 indicates the recovery of R-GB from Human Whole Blood spiked with 2 ng GB that was either frozen directly or separated first and then frozen.
- The recovery of R-GB from human whole blood spiked at either 2.5, 5, or 10 ng GB is presented in figure 8.
- The comparison of the recovery of R-GB from GB spiked & inhalation minipig whole blood is shown in figure 9.

Figure 6. Spiked Human Whole Blood

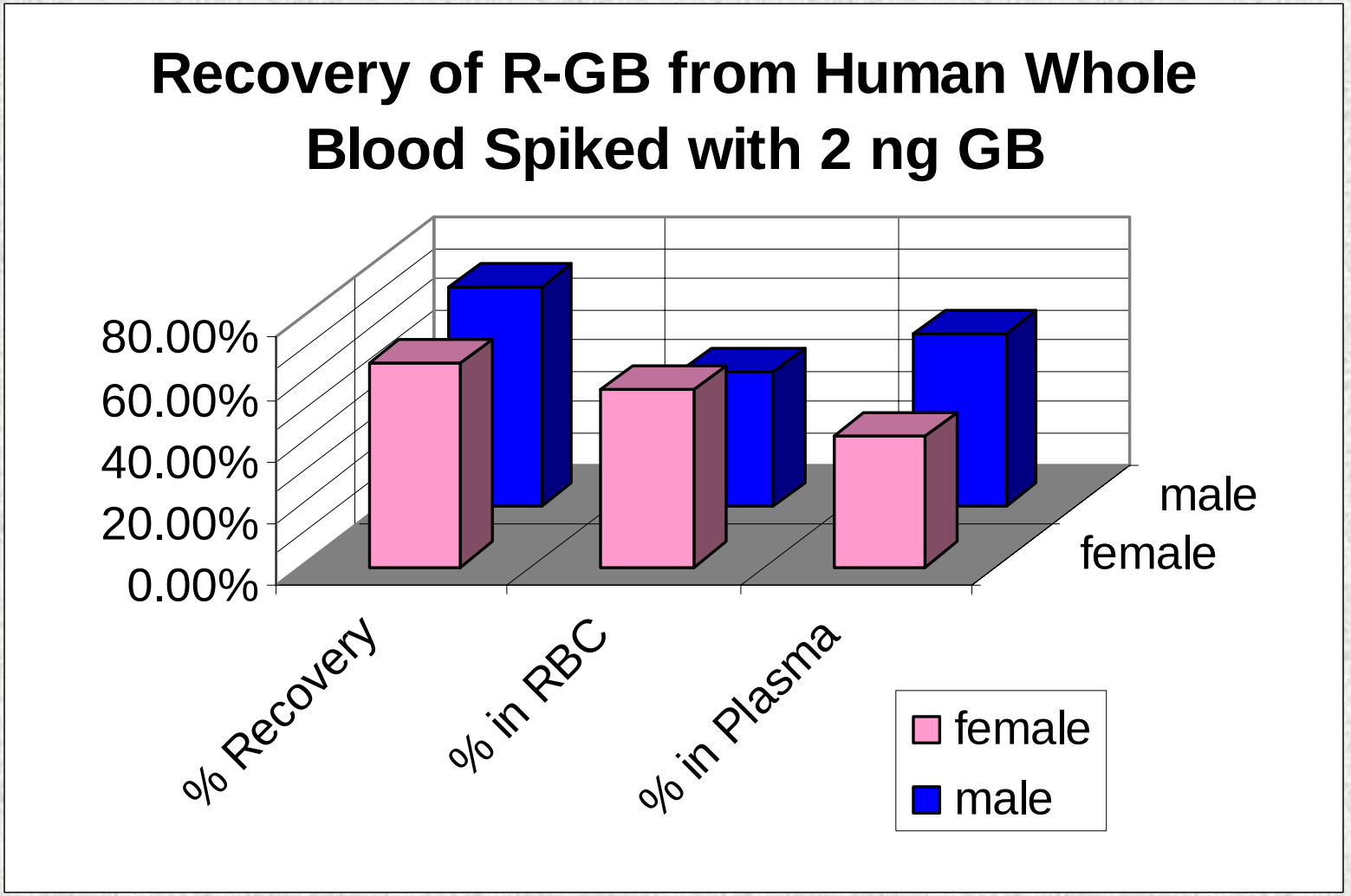


Figure 7. Fresh versus Frozen

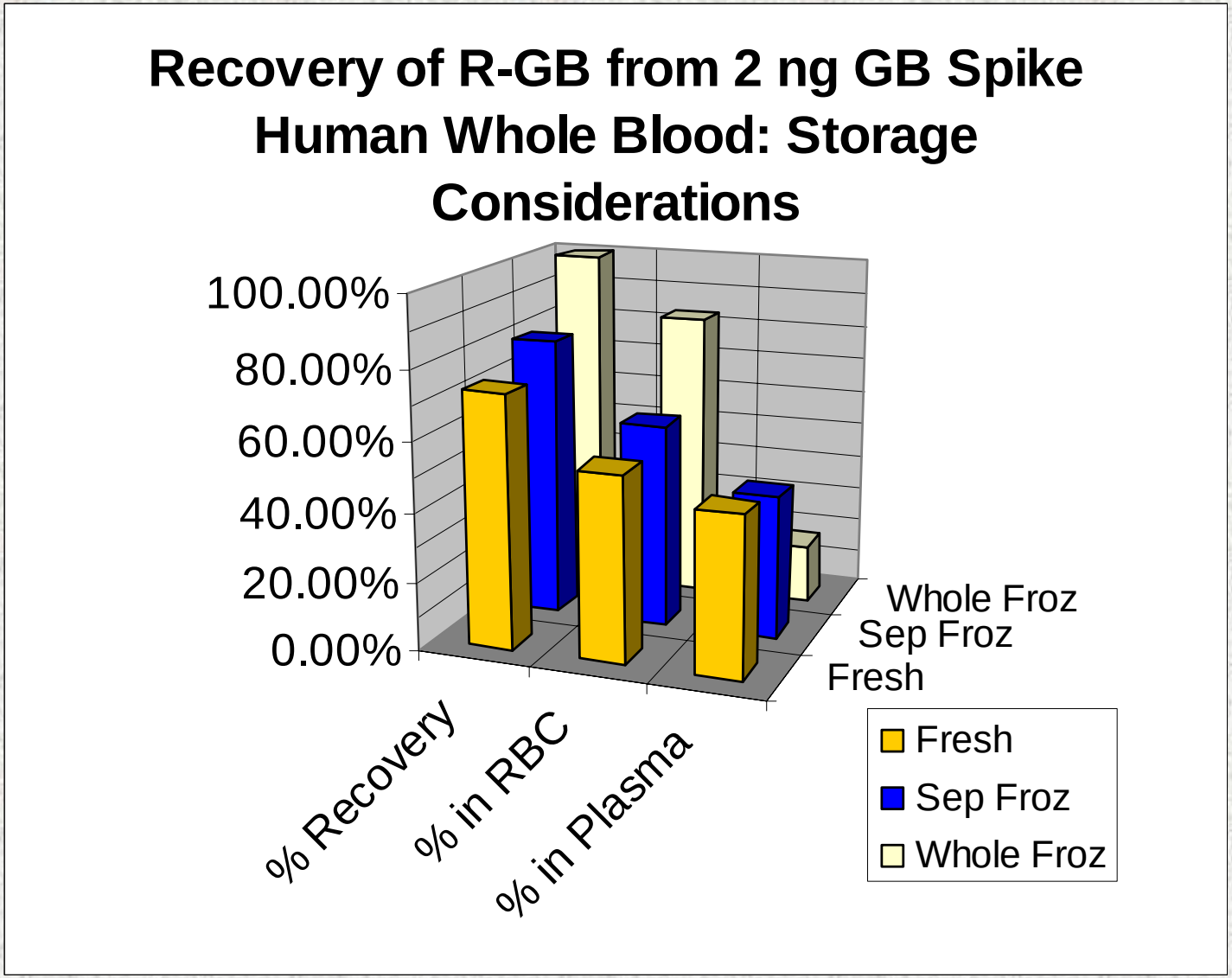


Figure 8. Spiked Human Whole Blood

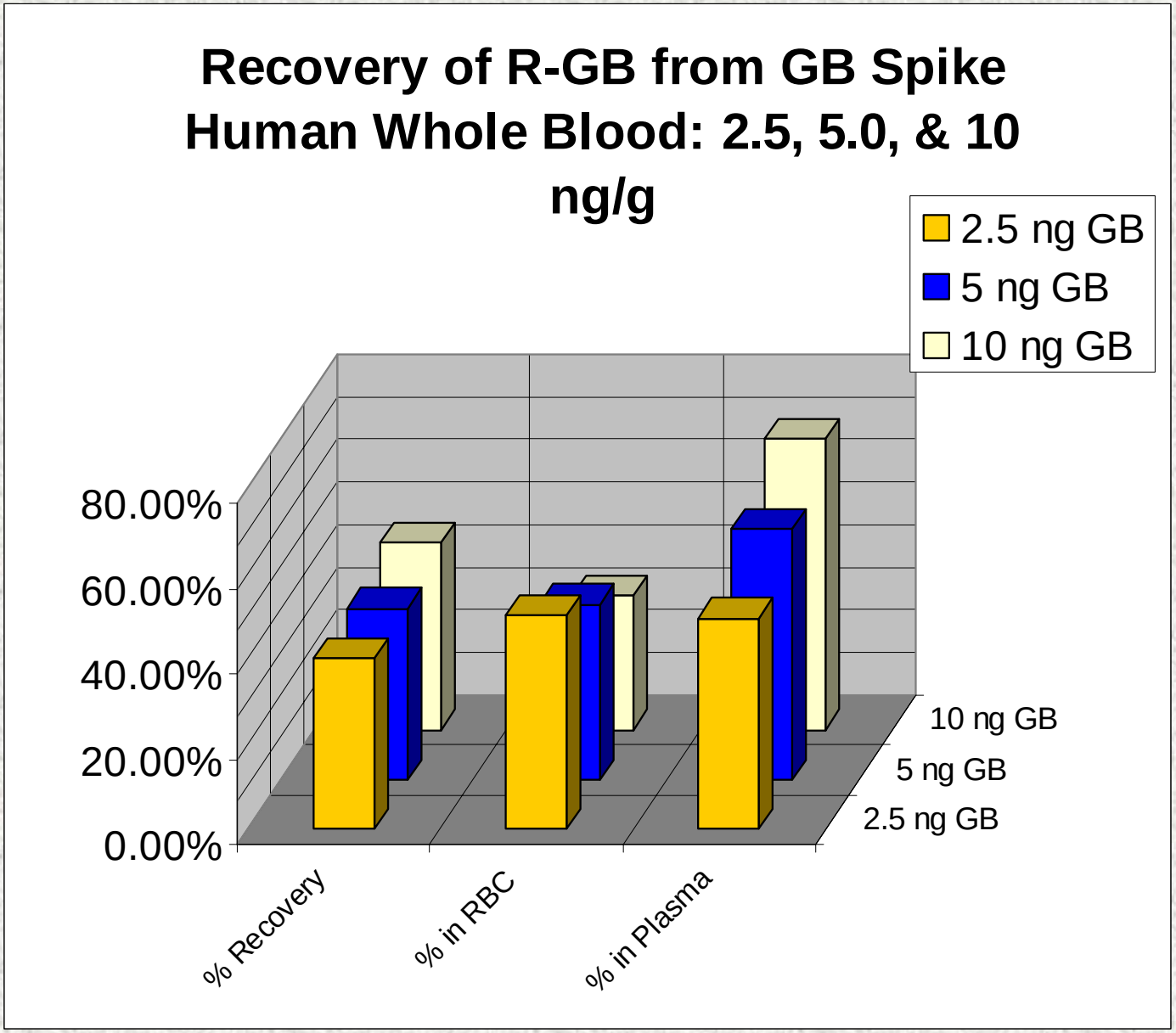
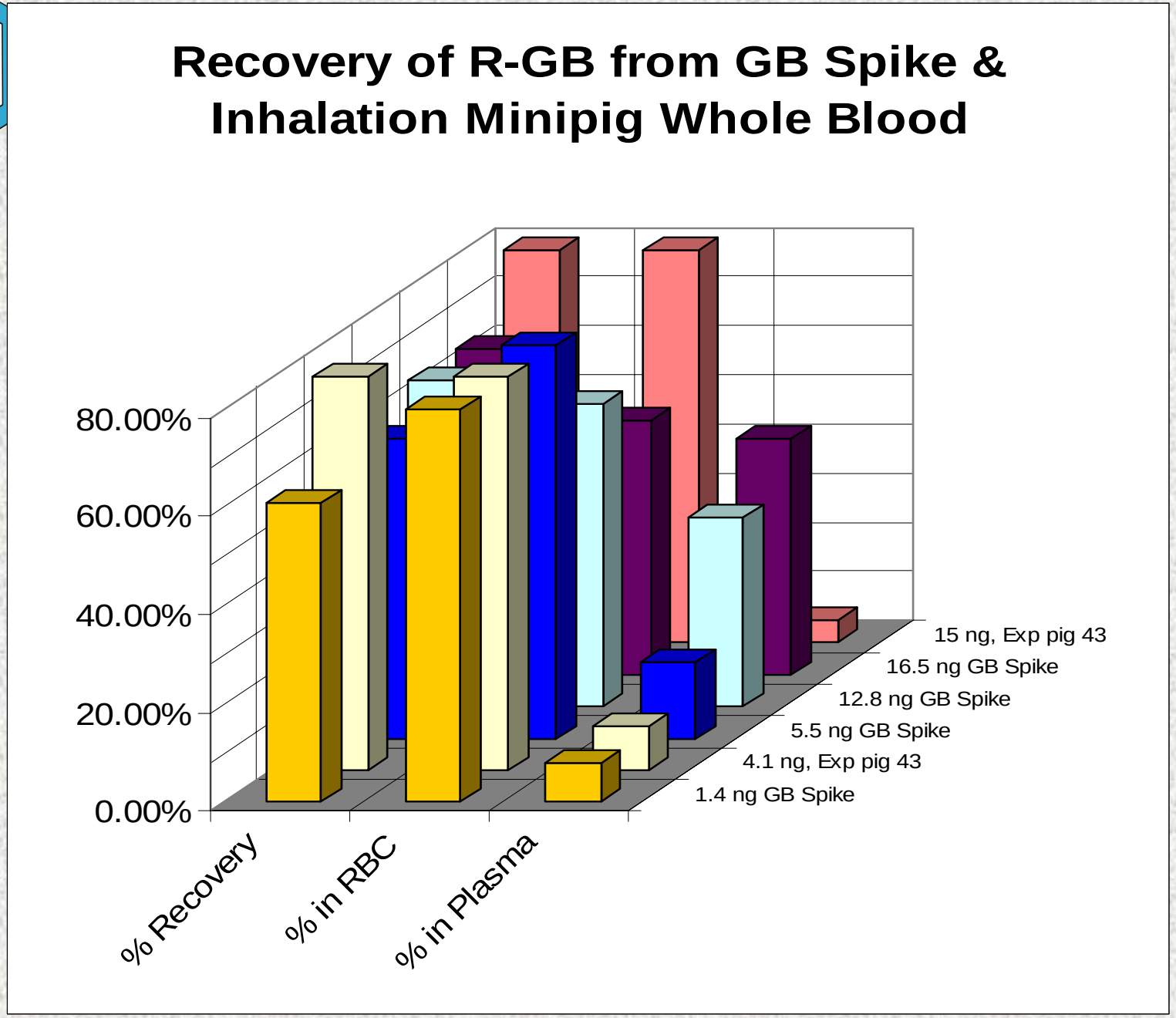


Figure 9. Minipig Blood



Summary/Conclusions

- The isotope dilution LVI method to determine GB in blood was superior to methods based on exterior calibration and non-mass spectrometric detection.
- Ammonia CI demonstrated increased sensitivity over EI. The automated LVI minimized sample preparation and improves sensitivity and precision.
- Results indicated the ability to quantify GB down to 200 pg/mL of extract despite the complexity of the red blood cell matrix.
- Conditions that needed to be optimize for the LVI included injection volume, initial temperature, initial time, pressure, and flow rate.

Summary/Conclusions

- The 14%-Cyanopropylphenyl 86%-Dimethylpolysiloxane (Rtx-1701) column performed better than the 5%-Diphenyl 95%-Dimethylpolysiloxane (HP-5ms) column.
- For human blood exposed to concentrations below 2.5 ng/g GB and for minipig blood at all exposure levels tested to date the majority of the GB that can be regenerated resides in the red blood cell fraction.
- Spiking minipig blood at high levels (near LCt_{50}) does not yield a realistic RBC/plasma split in R-GB levels
- Freezing the RBC fraction does not appear to effect R-GB production
- Freezing whole blood after exposure does not decrease overall recovery.