

# ***Challenges in Performing a Scientifically Meaningful Lipemic Plasma Test in Bioanalytical Method Validation***

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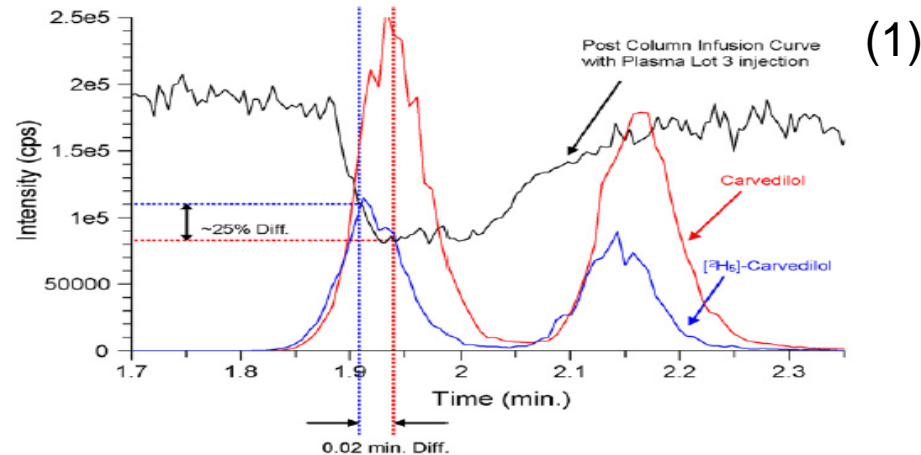
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# Bioanalytical Guidelines

- In 2003, **ANVISA** was the first regulatory agency to mention the inclusion of a lipemic matrix lot when validating an analytical method in plasma or blood (as part of selectivity).
- Only recently **EMA** has included performing matrix effect on lipemic plasma as part of their draft BMV guideline (2009) and guideline (Feb 2012).
- **Health Canada** follows EMA guidelines
- **ANVISA** included it in their new BMV guidance issued in May 2012 (for both selectivity and matrix effect).
- **FDA** has started focusing their attention on this subject during inspections as well.

# Method Development Challenges

- Lipids can potentially cause **matrix effect** and **recovery issues**.
- Using a **stable isotope-labeled internal standard** can often compensate for these effects, but it is not always the case.



- Currently, the type of commercially available "lipemic" plasma to be used for bioanalysis remains unclear.
  - **Differences between them are not well known.**
- Healthy volunteers are generally taken for clinical studies
  - There is **no precise definition** of what a "**healthy volunteer**" is in terms of lipids in blood, although the **Triglycerides** and **Cholesterol** concentrations are sometime used for the screening.

(1) Wang S, Cyronak M, Yang E. Does a stable isotopically labeled internal standard always correct analyte response? A matrix effect study on a LC/MS/MS method for the determination of carvedilol enantiomers in human plasma. *J Pharm Biomed Anal.* 2007 Jan 17;43(2):701-7. Epub 2006 Sep 7<sup>th</sup>.

# Types of "*Lipemic*" Plasma

## Postprandial lipemic plasma

- Collected after a **high-fat meal**
- Definition of high-fat meal by the **EMA Revised Guideline on the Investigation of Bioequivalence** (CPMP/QWP/EWP/1401/98 Rev. 1, EMA, 2010):

*"...the meal should be high fat (approximately **50%** of total caloric content of the meal) and high-calorie (approximately **800 to 1000 kcal**) meal. This test meal should derive approximately **150**, **250**, and **500-600** kcal from **protein**, **carbohydrate**, and **fat**, respectively. The composition of the meal should be described with regard to protein, carbohydrate and fat content (specified in grams, calories and relative caloric content (%))"*

# Types of "*Lipemic*" Plasma

## Plasma from high-triglycerides donors

- Visually-determined as lipemic plasma

## Artificial lipemic plasma

- **Lipisol™** fat emulsion mixed with plasma
- **Intralipid™** fat emulsion mixed with plasma

## Questions:

- *Which of these commercially available plasma and plasma mixes are the appropriate choice for bioanalysis?*
- *Should they all truly be defined as lipemic plasma?*

# Definition of Lipemic Plasma

- There is **no** a generally accepted definition
- **Definition of lipemia** proposed by Roche<sup>(1)</sup>:

*“Lipemia is defined as turbidity in serum samples which is visible to the **naked eye**. This is **usually** observed for triglycerides concentrations above 3.4 mmol/L (300 mg/dL).”*

- **Visual estimation** is a poor indication of lipemia
  - It is subjective and other factors than lipemia can influence the turbidity of samples
  - The degree of light scattering depends on the number, size and refractive index of suspended lipid particles.

(1) *Serum Indices: Reduction of clinical errors in laboratory medicine*, cobas® brochure, Roche, 2007. Available on-line: <http://www.roche-diagnostics.cz>

# Definition of Lipemic Plasma

## Lipemia index

### Pros

- Can be determined optically using automated, high-throughput analyzers
- Is correlated directly to the triglycerides concentrations
- Provides a good an evaluation of the lipids content

### Cons

- Is it really the best indicator for lipemic plasma for bioanalytical applications (BMV – LC-MS assays)?

# Study Design

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## Samples Analyzed

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20 regular plasma lots

3 lots from postprandial donors

3 lots from high-triglycerides donors

8 synthetic lipemic plasma mixes

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## Evaluations Performed

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Triglycerides concentration

Total cholesterol concentration

Lipemia index

Phospholipids (Normalized)

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Natural  
lipemic  
plasma



# Study Design

**Regular plasma** lots were divided in two groups:

- “**Normal**” plasma lots, which are defined as those in the normal concentration ranges for triglycerides ( $<1.70$  mM)
- “**Abnormal**” plasma lots, which are defined as those **outside** of the normal concentration ranges for triglycerides ( $\geq 1.70$  mM)
- $1.70$  mM ( $150$  mg/dL) value is from the guidelines of the **American Heart Association**.
- [http://www.heart.org/HEARTORG/GettingHealthy/NutritionCenter/Triglycerides\\_UCM\\_306029\\_Article.jsp](http://www.heart.org/HEARTORG/GettingHealthy/NutritionCenter/Triglycerides_UCM_306029_Article.jsp)

# Results

## Normal Plasma Lots (n = 14)

### *Regular Lots Within Normal Triglycerides Range*

|         | Lipemia Index | Total Cholesterol (mM, Normal is < 5.2) | Triglycerides (mM, Normal is < 1.70) | Phospholipids (Normalized) |
|---------|---------------|---|--------------------------------------|----------------------------|
| Average | 23            | 4.1                                     | 1.03                                 | 1.00                       |
| CV%     | 61.8          | 27.8                                    | 30.8                                 | 20.3                       |

## Abnormal Plasma Lots (n = 6)

### *Regular Lots Outside Normal Triglycerides Range*

|         | Lipemia Index | Total Cholesterol (mM, Normal is < 5.2) | Triglycerides (mM, Normal is < 1.70) | Phospholipids (Normalized) |
|---------|---------------|---|--------------------------------------|----------------------------|
| Average | 79            | 4.6                                     | 2.26                                 | 1.20                       |
| CV%     | 62.3          | 19.9                                    | 8.3                                  | 14.3                       |

## All Regular Lots (n = 20)

|         | Lipemia Index | Total Cholesterol (mmol/L, Normal is < 5.2) | Triglycerides (mmol/L, Normal is < 1.70) | Phospholipids (Normalized) |
|---------|---------------|---|--|----------------------------|
| Average | 40            | 4.2   | 1.40                                     | 1.06                       |
| CV%     | 95.4          | 25.4  | 45.8                                     | 21.6                       |

# Results

## Postprandial Donors

| Sample Name | Lipemia Index | Total Cholesterol (mM, Normal is < 5.2) | Triglycerides (mM, Normal is < 1.70) | Phospholipids (Normalized) |
|-------------|---------------|---|--------------------------------------|----------------------------|
| 1           | 42            | 3.9                                     | <b>1.87</b>                          | 1.14                       |
| 2           | 68            | 3.2                                     | 1.56                                 | 0.85                       |
| 3           | 136           | 4.8                                     | <b>3.80</b>                          | 1.54                       |
| Average     | 82            | 4.0                                     | 2.41                                 | 1.18                       |
| CV%         | 59.2          | 20.2                                    | 50.4                                 | 29.5                       |

## High-Triglycerides Donors

| Sample Name | Lipemia Index | Total Cholesterol (mM, Normal is < 5.2) | Triglycerides (mM, Normal is < 1.70) | Phospholipids (Normalized) |
|-------------|---------------|---|--------------------------------------|----------------------------|
| 4           | 58            | 2.9                                     | <b>1.79</b>                          | 1.08                       |
| 5           | 124           | <b>5.2</b>                              | <b>3.06</b>                          | 1.22                       |
| 6           | 207           | 4.3                                     | <b>5.30</b>                          | 1.86                       |
| Average     | 130           | 4.1                                     | 3.38                                 | 1.38                       |
| CV%         | 57.6          | 28.0                                    | 52.5                                 | 30.0                       |

## Normal Plasma Lots (n = 14)

### *Regular Lots Within Normal Triglycerides Range*

|         | Lipemia Index | Total Cholesterol (mM, Normal is < 5.2) | Triglycerides (mM, Normal is < 1.70) | Phospholipids (Normalized) |
|---------|---------------|---|--------------------------------------|----------------------------|
| Average | 23            | 4.1                                     | 1.03                                 | 1.00                       |
| CV%     | 61.8          | 27.8                                    | 30.8                                 | 20.3                       |

# Results

## Normal Plasma Lots (n = 14)

### Regular Lots Within Normal Triglycerides Range

|         | Lipemia Index | Total Cholesterol (mM, Normal is < 5.2) | Triglycerides (mM, Normal is < 1.70) | Phospholipids (Normalized) |
|---------|---------------|---|--------------------------------------|----------------------------|
| Average | 23            | 4.1                                     | 1.03                                 | 1.00                       |
| CV%     | 61.8          | 27.8                                    | 30.8                                 | 20.3                       |

## Lipisol™ 10% : Normal Plasma

| % v/v | Lipemia Index | Total Cholesterol (mM, Normal is < 5.2) | Triglycerides (mM, Normal is < 1.70) | Phospholipids (Normalized) |
|-------|---------------|---|--------------------------------------|----------------------------|
| 50:50 | 694           | 3.5                                     | 13.6                                 | 1.23                       |

## Intralipid™ 20% : Normal Plasma

| % v/v | Lipemia Index | Total Cholesterol (mM, Normal is < 5.2) | Triglycerides (mM, Normal is < 1.70) | Phospholipids (Normalized) |
|-------|---------------|---|--------------------------------------|----------------------------|
| 0:100 | 12            | 2.8                                     | 0.5                                  | 0.71                       |
| 1:99  | 204           | 2.8                                     | 5.2                                  | 0.82 <sup>1</sup>          |
| 2:98  | 392           | 2.8                                     | 10.4                                 | 0.89 <sup>1</sup>          |
| 4:96  | 762           | 2.8                                     | 19.3                                 | 1.02 <sup>1</sup>          |
| 5:95  | 972           | 2.8                                     | 25.1                                 | 1.09 <sup>1</sup>          |
| 25:75 | 3366          | 2.3                                     | 122.8                                | 2.52                       |
| 50:50 | 8952          | 3.1                                     | 242.4                                | 4.08                       |
| 100:0 | 18016         | 5.1                                     | 485.2 <sup>2</sup>                   | 5.44                       |

1: Data obtained from a linear interpolation (3 data points,  $R^2 = 0.9980$ ).

2: Data obtained from a linear extrapolation (7 data points,  $R^2 = 1.0000$ ).

# Results

1. On average, the **high-triglycerides donors** lots tested contained more triglycerides and more phospholipids than **post-prandial plasma** lots.
2. Large **inter-lots variability** are obtained for triglycerides and phospholipids content of the **post-prandial** donors and **high-triglycerides** donors lots. Neither of these methods (**high-fat meal & visual estimation**) is sufficiently reliable by itself to be used to consistently generate plasma which has an adequate minimum level of lipemia.

Plasma lots should be tested prior to usage to ensure that the desired minimum level of lipemia is achieved. **How?**

# Results

3. The **cholesterol** concentration was similar in all cases and it appears to be fairly independent of other lipids.

**Cholesterol should not be part of lipemic plasma test**

If it is determined to have an impact for a given method, this should be addressed as part of method development and BMV by taking measures deemed scientifically sound on a case-by-case basis

4. **Synthetic plasma mixes are** not sufficiently concentrated in phospholipids to be spiked in small volumes (**1-2% v/v**) in regular plasma, which is desirable to avoid **overdiluting** the matrix.

**Synthetic plasma mixes are not recommended for lipemic plasma test in BMV**

# Results

- The triglycerides concentration is strongly correlated to
  - Total phospholipids content (**73%**) and to
  - Lipemia index (**89%**)

## Triglycerides concentration

is the best indicator of overall lipemia among the variables tested here

How much?

## Pearson Correlation Coefficient (r)

| Variables     | Lipemia index | Cholesterol | Triglycerides | Phospholipids |
|---------------|---------------|-------------|---------------|---------------|
| Lipemia index |               | 0.29        | 0.89          | 0.64          |
| Cholesterol   | 0.29          |             | 0.25          | 0.65          |
| Triglycerides | 0.89          | 0.25        |               | 0.73          |
| Phospholipids | 0.64          | 0.65        | 0.73          |               |

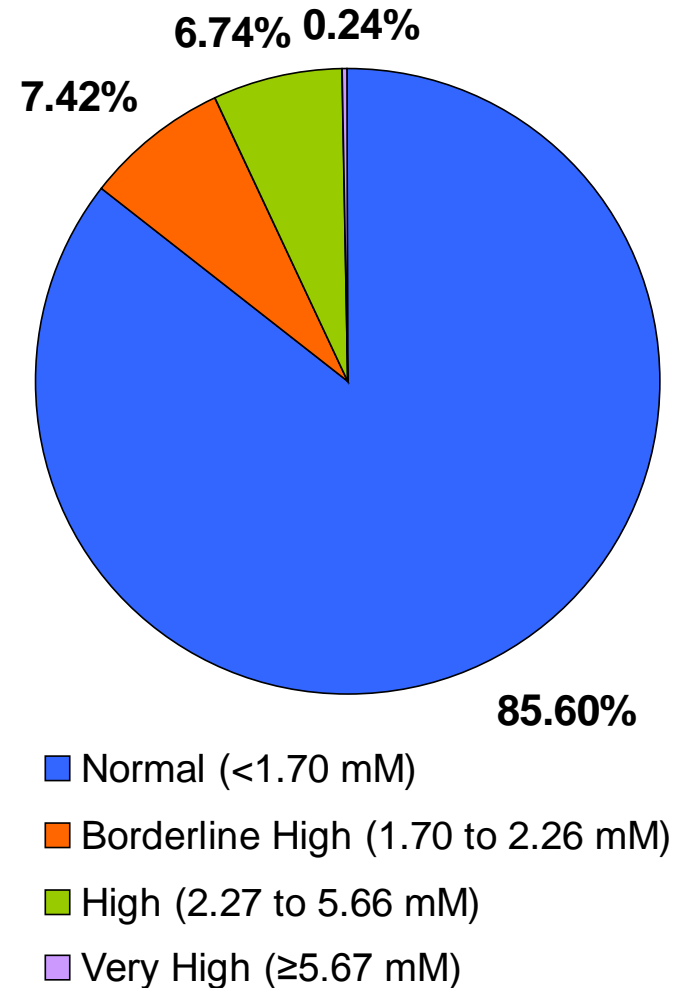
$0.0 < |r| < 0.3$  weak correlation

$0.3 < |r| < 0.7$  moderate correlation

$|r| > 0.7$  strong correlation

# Additional Results – Choosing the Minimum Triglycerides Level to be Used

- The choice of the **minimum triglycerides level** of the lipemic plasma to be used for BMV should be based on both:
  - **Matrix availability** and
  - **Statistical data on sample lipemia.**
- **Triglycerides concentration of 2952 volunteers** from our clinic was compiled to provide those statistics.





# Additional Results – Choosing the Minimum Triglycerides Level to be Used

- A value of **3.4 mM (300 mg/dL)** should not cause matrix availability issues and allow to cover **98.24%** of samples.

| Minimum Triglycerides Concentration (mM) | Minimum Triglycerides Concentration (mg/dL) | Number of Samples | Percentage of Total Samples |
|--|---|-------------------|-----------------------------|
| 2.26                                     | 200   | 207               | 7.01                        |
| 2.38                                     | 210   | 177               | 6.00                        |
| 2.49                                     | 220   | 155               | 5.25                        |
| 2.60                                     | 230   | 135               | 4.57                        |
| 2.72                                     | 240   | 119               | 4.03                        |
| 2.83                                     | 250   | 103               | 3.49                        |
| 2.94                                     | 260   | 91                | 3.08                        |
| 3.06                                     | 270   | 75                | 2.54                        |
| 3.17                                     | 280   | 66                | 2.24                        |
| 3.28                                     | 290   | 60                | 2.03                        |
| 3.40                                     | 300   | 52                | 1.76                        |
| 3.96                                     | 350   | 29                | 0.98                        |
| 4.53                                     | 400   | 20                | 0.68                        |
| 5.66                                     | 500   | 7                 | 0.24                        |

# Conclusions

- Plasma lots from **high-triglycerides** and **postprandial donors**
  - have a high content in triglycerides & phospholipids,
  - but a large inter-lot variability is obtained
- **Natural lipemic plasma with a high triglycerides level best reflects lipemic incurred samples** obtained as part of clinical trials

## NOTE:

*Evaluation of lipids in plasma **is not done systematically** on all subject enrolled; a volunteer can be enrolled as a **healthy volunteer** and present a **disorder in triglycerides level.***

# Recommendation

In order to certify that a given lot of this matrix can be used to make a

**Scientifically Meaningful Lipemic Plasma Test**  
for BMV,

we recommend to certify its triglycerides level to ensure that it meets a

**“Minimum Concentration of 3.4 mM (300 mg/dL)”**

*Based on our data, this approach would allow **covering most samples (98.24%)** while also ensuring **good matrix availability***