# A RAPID LIQUID CHROMATOGRAPHIC METHOD WITH EVAPORATIVE LIGHT SCATTERING DETECTION FOR POSTMORTEM GLUCOSE MEASUREMENT IN VITREOUS HUMOR

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**ABSTRACT**

Hyperglycemia is considered a modifiable risk factor that worsens the prognosis of acute myocardial infarction. Accurate glucose measurement is crucial for assessing hyperglycemia in patients who have died from infarction. Since postmortem blood glucose levels are unreliable due to metabolic changes, vitreous humor (VH) provides a reliable alternative for glucose analysis. In this study, a simple and rapid liquid chromatographic method with evaporative light scattering detection (LC-ELSD) was developed and validated for glucose quantification in VH, including a stability study. The method was developed using a Diol column with an isocratic mobile phase consisting of water and acetonitrile (23:77 v/v) at a flow rate of 1 mL/min, achieving a glucose retention time of 8.3 minutes. The column temperature was maintained at 40°C, while the ELSD evaporation temperature was set at 45°C. Validation was performed according to FDA guidelines. Calibration curves demonstrated linearity over a range of 15–300 mg/dL with $R^2 > 0.99$. Intraday and interday precision and accuracy were within ±15% of nominal values, except at the LLOQ, where they were within ±20%. The extraction recoveries ranged from 97.82 to 102.66%. The method exhibited high selectivity and specificity, with no interference from endogenous compounds or co-administered drugs. Stability studies confirmed that glucose remained stable in VH for up to 72 hours at -20°C, +4°C, and room temperature. The results demonstrate that the proposed method offers a reliable and efficient approach for glucose determination in VH suitable for forensic applications, eliminating the need for derivatization.

**Keywords:** Glucose, Postmortem, Validation, Vitreous Humor.

## 1. INTRODUCTION

Current evidence suggests a relationship between hyperglycemia and acute myocardial infarction. Elevated plasma glucose levels have been identified as mediators that worsen the prognosis of infarction and increased the risk of death. Therefore, hyperglycemia during a myocardial infarction could be a significant and potentially modifiable risk factor for adverse outcomes. Managing blood glucose levels is crucial for improving the prognosis and overall outcomes for these patients [1-4]. To assess hyperglycemia in patients who have died from infarction, accurate glucose measurement is essential. Since postmortem blood glucose concentrations have no diagnostic value due to alterations in glucose metabolic pathways after death, vitreous humor (VH) represents a reliable alternative for glucose determination. VH is better preserved than blood after death, as it is protected from postmortem degradation and contamination due to its isolated localization and very low cell content. The autolytic processes in VH are slower compared to those in blood [5-7]. Based on clinical observations, the concentration of glucose in VH is about half that in blood. Thus, a glucose value of 10 mmol/L in a vitreous sample collected about one day or more after death would theoretically correspond to an antemortem blood glucose level of approximately 20 mmol/L [7,8]. VH is a colorless, jelly-like, hydrophilic gel located within the vitreous body of the eye, with an approximate volume of 4–5 mL. It is composed of approximately 99% water with trace amounts of collagen, hyaluronic acid, sugars, urea, creatinine, and electrolytes such as sodium, potassium, chloride, calcium, and magnesium [8].

Glucose concentrations are mainly determined photometrically through enzymatic reactions, particularly using hexokinase and glucose oxidase methods, in whole blood, serum or plasma, especially for the diagnosis and management of patients with diabetes [3,9,10]. Postmortem glucose determination in VH is less frequent [5-7,11] and is primarily used to detect glucose metabolism disorders or diabetic complications [5-7]. Chromatographic analysis of glucose in biological fluid is less common. Some studies have quantified glucose using LC-UV after derivatization in human serum for cancer detection [12-13], and in dog serum for determination of glucose before and after eating a meal [14]. Additionally, other studies report an LC-MS/MS method applied to rat plasma as a biomarker of some diseases [15] and UPLC-MS/MS methods for quantification of glucose in plasma of diabetic patients [16], and for the quantification of different sugars and sweeteners in human urine [17].

In this work, we present an LC method with evaporative light scattering detection (ELSD) for glucose determination, offering a simpler alternative to UV detection as it does not require derivatization. ELSD response is independent of the chemical structures of the compounds. Some analytical methods using LC-ELSD for glucose quantification have been reported, primarily applied to the evaluation of carbohydrates in food samples and plants [18-20]. However, to our knowledge, no LC-ELSD methods for glucose quantification in VH have been reported. Therefore, the aim of this work was to develop and validate an LC-ELSD method for glucose determination in VH, including a stability study.

## 2. EXPERIMENTAL

### 2.1 Materials and reagents

Glucose standard (≥ 99.5% purity) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN) LC grade was obtained from Merck (Darmstadt, Germany). Milli-Q grade water was used for the preparation of the mobile phase (Milli Q® system, Merck/Millipore, Molsheim, France).

### 2.2 Instrumentation

Chromatographic analysis was performed on a YL9100 LC system (Young Lin Instrument, Anyang, Korea). The system consisted of a YL9110 quaternary pump, a YL9101 vacuum degasser, a YL9130 column compartment, and a Sedex model 85 LT-ELSD detector (low temperature evaporative light scattering detector) (Sedere S.A., Alfortiville Cedex, France). It was equipped with a manual injector and a 20 µL loop. Instrument control and data collection were performed using YL-Clarity software, version 3.0.4.444.

### 2.3 Chromatographic conditions

The separation was performed using a Kromasil® 60-5Diol column (250 mm × 4.6 mm, 5 µm; Nouryon, Bohus, Sweden). The mobile phase consisted of water and ACN, 23:77 v/v, in isocratic elution mode, with a 1 mL/min flow rate. The column temperature was set at 40°C, while the ELSD evaporation temperature was set at 45°C. The gain was set to 7, and the nebulizer gas pressure was maintained at 3 bar. The optimal proportion of water and ACN in the mobile phase, as well as the optimal column temperature, evaporation temperature, and flow rate, were determined using an experimental design approach with Statgraphics Centurion XV, version 15.2.05.

### 2.4 Preparation of standards and quality controls

A stock solution of glucose was prepared in water at a concentration of 1000 mg/dL. This solution was used to spike the aliquots of drug-free human VH samples, resulting in six calibration standards with glucose concentrations of 15, 30, 50, 100, 150, 200, 250, and 300 mg/dL. Quality controls samples (QC) were independently prepared following the same procedure as the calibration standards, yielding four concentration levels (LLOQ, low, medium, and high): 15, 30, 150, and 300 mg/dL. The stock solution was stored in glass vials at −20 °C. Calibration standards and QC samples were freshly prepared before each analysis.

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### 2.5 Collection and sample preparation

VH was obtained during medico-legal autopsies (Legal Medical Service of Concepción, Chile) with a time since death of two days or less, using 20-gauge needles. A total of 2-3 mL of VH was collected from the center of the eyes using a syringe. The collected samples were stored frozen at −20°C in polypropylene containers until analysis. Study approval was obtained from the LMS.

Before analysis, samples were thawed at room temperature, vortexed for 10 seconds, and centrifuged at 3000 rpm for 15 minutes. Only the supernatant was used for analysis.

### 2.6 Method validation

The method was validated according to the Food and Drug Administration (FDA), Bioanalytical Method Validation guidance [21]. Linearity, sensitivity, accuracy, precision, extraction recovery, carryover, selectivity, specificity, and stability were used as the validation parameters.

#### 2.6.1 Linearity and sensitivity

The selected concentration range for the analytical method was based on glucose levels typically observed during hyperglycemia. Calibration curves were constructed using six non-zero calibration standards with concentrations of 15, 30, 50, 100, 150, 200, 250, and 300 mg/dL. Linearity was determined by plotting peak area against the glucose concentration. Each solution was injected three times, and three independent calibration curves were analyzed in VH. The back-calculated standard concentrations were determined to meet the acceptance criteria: calibration standards should be within ± 15% of nominal (theoretical) concentrations, except at LLOQ, where the calibrator should be within ± 20 % of the nominal concentrations. This criterion should be met for at least 75% of the calibration standards. Linearity was statistically evaluated by linear regression using analysis of variance (ANOVA). The LLOQ was defined as the lowest concentration on the calibration curve.

#### 2.6.2 Accuracy and precision

Intraday accuracy and precision were assessed by performing five independent assays for each QC level (LLOQ, low, medium, and high) on the same day under the same experimental conditions. Interday accuracy and precision were evaluated by carrying out the assays on three different days. Accuracy was calculated as relative error (RE, %) (measured concentration-nominal concentration)/nominal concentration × 100), and the precision was expressed as the relative standard deviation (RSD, %). To meet the acceptance criteria, accuracy and precision had to be within ± 15% of the nominal value, except at the LLOQ, where a tolerance of ± 20% was applied.

#### 2.6.3 Recovery

The extraction recovery from VH samples was determined by comparing the responses of QC samples spiked before extraction with those spiked after extraction which represent 100 percent recovery, at low, medium, and high QC levels. Recovery does not need to be 100%, but the results should be consistent and reproducible.

#### 2.6.4 Selectivity, specificity and carryover

Blank human VH samples from 10 deceased individuals were used to evaluate selectivity. Specificity was assessed by analyzing potential concomitant medications commonly administered in cases of acute myocardial infarction, to ensure that no interfering peaks were present at the glucose retention time. Any interfering compounds detected should exhibit a response of less than 20% at the LLOQ.

Carryover was assessed by injecting blank samples immediately after the highest calibration standard concentration (300 mg/dL). To meet acceptance criteria, carryover should not exceed 20% of the LLOQ.

#### 2.6.6 Stability studies

Short-term stability at high, medium, and low concentrations was evaluated in VH at room temperature (23 ± 2°C), +4 ± 2°C, and -20 ± 2°C for up to 72 h. Samples were analyzed after thawing for 1 hour at room temperature if stored at -20°C, or after a few minutes if stored at 4°C. Stability was determined by comparing the concentration at each time point with the initial concentration (t0), with each concentration analyzed in triplicate. To meet acceptance criteria, results should be within ± 15% of the initial nominal concentration.

## 3. RESULTS AND DISCUSSION

### 3.1 Analytical method development and optimization

Four factors were evaluated to assess their effects on the ELSD detection of glucose: ACN proportion in the mobile phase (70-85%), column temperature (35-45°C), evaporation temperature (35-45°C) and flow rate (0.8-1.2 mL/min). A two-level full factorial design ($2^4$) with a central point and two replicates was employed. The peak height (response) was measured for each experimental condition.

Optimal chromatographic conditions, which provided good peak shape and appropriate resolution between glucose and endogenous compounds within a short run time, were established using a mobile phase composed of 77% ACN and 23% water, with a 1 mL/min flow rate on a Kromasil® 60-5Diol column (250 mm × 4.6 mm, 5 µm). Under these conditions, the total run time was 10.0 min, enabling high-throughput sample processing. The retention time for glucose was observed at 8.3 min. System suitability parameters met acceptable criteria: Resolution > 2.0, peak tailing factor 1.1, and theoretical plates 2300. Figure 1 shows chromatograms of the standard solution (A) and a blank sample spiked with glucose at 200 mg/dL (B), obtained using the optimized HPLC method.

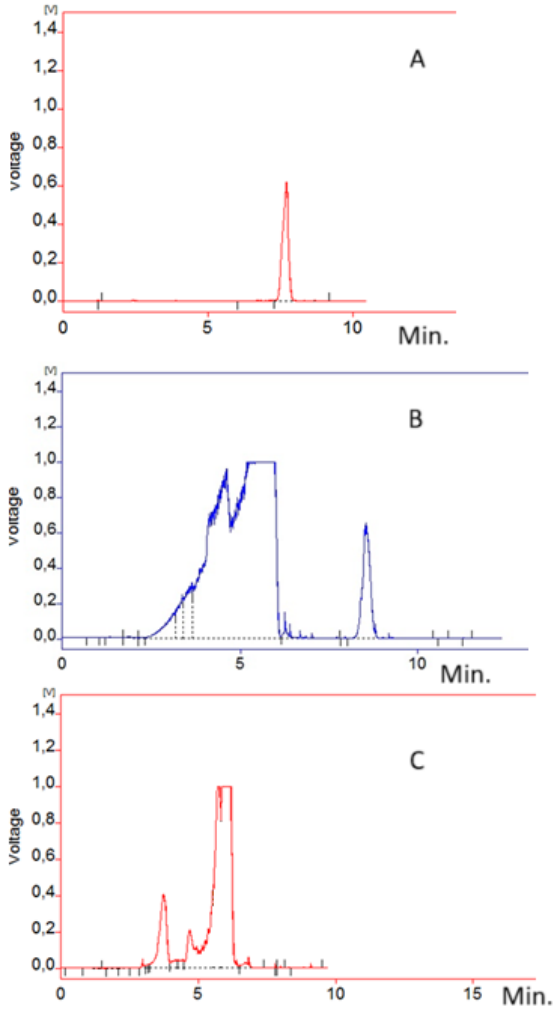


**Figure 1:** Chromatogram of standard solution at 200 mg/dL (A), blank VH sample spiked with glucose at 200 mg/dL (B), and blank VH sample (C). Column, Kromasil® 60-5Diol; mobile phase, ACN and water, 77:23 v/v; detection, ELSD; flow rate, 1,0 mL /min.

### 3.2 Sample preparation

The VH samples were thawed at room temperature, centrifuged at 3000 rpm for 10 minutes, and the supernatant was used without any additional treatment. This approach yielded chromatograms free from interference by endogenous compounds, as demonstrated in the selectivity study. The sample preparation method used in this study is simpler, faster, and more cost-effective than previously published methods, which are often more complex and time-consuming. These include enzymatic digestion with hyaluronidase, heat liquefaction at 100 °C for approximately 5 minutes, and solvation via ultrasound in an ultrasonic bath at 20°C for 15 minutes, all followed by centrifugation for 10 minutes prior to analysis [11].

Due to the high viscosity of VH, analytical problems such as imprecision, inaccuracy, and variability in compound analysis have been reported, depending on the sample treatment applied. Therefore, reducing viscosity through sample pre-treatment is essential. Similar mean glucose values have been found when comparing the previously mentioned treatments (hyaluronidase, heat, ultrasound) with centrifugation alone [11, 22], supporting the validity of the sample preparation method used in this study. When analyzing vitreous humor, it is important to consider that glucose values decline during the early postmortem period (within the first 24h), likely due to consumption by retinal cells and hyalocytes. However, after their death, glucose levels tend to stabilize for at least three days [23].

### 3.3 Method Validation

#### 3.3.1 Linearity and sensitivity

A linear correlation was obtained between peak area and glucose concentrations over the range of 15-300 mg/dL. All three calibration curves demonstrated good linearity, with determination coefficient ($R^2$) higher than 0.99. The typical equation for the calibration curve was y= 57.43x – 825.61. All calibration standards meet the acceptance criteria. Statistical analysis by ANOVA confirmed the linearity of the curves, with $p < 0.005$.

The LLOQ was established at 15 mg/dL. Since postmortem vitreous glucose values above 91 mg/dL are highly suggestive of hyperglycemia prior to death [23], the LLOQ falls well below this threshold, making it suitable for the quantitative determination of glucose in VH.

#### 3.3.2 Accuracy and precision

The results of the accuracy and precision studies are presented in Table 1. The RE and RSD values for both intraday and interday accuracy and precision were within ± 15% of the nominal value, and within ± 20% at the LLOQ, confirming that the method is both accurate and precise.

**Table 1.** Intraday and interday accuracy and precision of glucose QC samples

| Nominal concentration (mg/dL) | Intraday[a] | | | Interday[b] | | |
|---|---|---|---|---|---|---|
| | Measured concentration (mg/dL) | RSD (%) | RE (%) | Measured concentration (mg/dL) | RSD (%) | RE (%) |
| 15 | 17.83 | 6.01 | 12.99 | 18.30 | 15.07 | 15.97 |
| 30 | 27.02 | 7.80 | -5.19 | 26.83 | 2.01 | -5.86 |
| 150 | 146.99 | 4.31 | -1.55 | 142.08 | 3.49 | -4.84 |
| 300 | 274.67 | 3.01 | -8.38 | 271.31 | 1.85 | -9.50 |

[a] Analyzed on the same day (n = 5)
[b] Analyzed on three different days (n = 15)

#### 3.3.3 Extraction recovery

The results of the extraction recovery study are presented in Table 2. According to the t-test (n = 9, $\alpha = 0.05$) the obtained recovery did not significantly differ from the expected value (100%), demonstrating that glucose was successfully recovered from VH. Furthermore, the RSD was less than 3.3%, indicating that the recovery was reproducible.

**Table 2.** Glucose extraction recoveries from VH

| Nominal concentration (mg/dL) | Extraction recovery | |
|---|---|---|
| | Mean ± SD[a] | RSD (%) |
| 30 | 97.82 ± 2.25 | 2.30 |
| 150 | 102.66 ± 3.35 | 3.26 |
| 300 | 102.65 ± 2.80 | 2.73 |

[a]n = 3

#### 3.3.4 Selectivity, specificity and carryover

The analysis of blank human VH samples demonstrated no interference from endogenous compounds at the glucose retention time, confirming the method's selectivity. Additionally, the analysis of potentially co-prescribed drugs in cases of acute myocardial infarction, including atorvastatin, acetylsalicylic acid, digoxin, and propranolol, showed no interference with the glucose peak, demonstrating the method's specificity. The retention times for atorvastatin, acetylsalicylic acid, digoxin and propranolol were 2.8, 2.4, 2.9 and 3.6 minutes, respectively. Figure 1 shows typical chromatograms of blank VH and blank VH spiked with glucose at 200 mg/dL.

The absence of carryover was confirmed by the lack of peaks in the chromatogram of a blank sample solution, analyzed immediately after the highest calibration standard.

#### 3.3.5 Stability studies

Short-term stability results demonstrated that glucose remained stable for at least 72 hours in VH samples stored at room temperature, +4 °C, and -20 °C, as the deviation from the nominal concentration was less than 15% under all conditions, ranging from 0.39% to 4.13% (Table 3). These results are consistent with previous studies [24], which reported that glucose remains stable for approximately 12 hours at room temperature, 2 to 6 days at 4°C, and several months when frozen at −21°C.

**Table 3.** Stability of Glucose in VH

| Time (h) | Degradation (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 30 (mg/dL) | | | 150 (mg/dL) | | | 300 (mg/dL) | | |
| | +23 (°C) | +4 (°C) | -20 (°C) | +23 (°C) | +4 (°C) | -20 (°C) | +23 (°C) | +4 (°C) | -20 (°C) |
| 24 | 2.95 | 1.77 | 0.98 | 2.25 | 2.00 | 1.06 | 2.07 | 1.50 | 0.51 |
| 72 | 3.74 | 3.34 | 0.39 | 4.13 | 3.26 | 1.29 | 3.87 | 1.70 | 0.67 |

## CONCLUSIONS

A novel LC-ELSD method was developed and validated for the quantification of glucose in VH, offering a reliable and efficient alternative for postmortem analysis. The method demonstrated excellent performance over the 15–300 mg/dL range, meeting all FDA validation criteria. Key advantages include simplified sample preparation requiring only centrifugation, simple LC-ELSD technique, leveraging the benefits of evaporative light scattering detection such as the elimination of the need for derivatization, and a short chromatographic run time, enabling high-throughput sample processing. Additionally, stability studies demonstrated that glucose remained stable under various storage conditions for up to 72 hours. This method is particularly suitable for forensic applications, especially in cases of acute myocardial infarction, where hyperglycemia represents a modifiable risk factor.

## DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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