



CHROMATOGRAM-BASED PREDICTORS OF HbA1c REPORTING FAILURE AND POSITIVE BIAS ON BIO-RAD HPLC IN SAMPLES WITH ABNORMAL HAEMOGLOBIN PEAKS

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Abstract

Background: Abnormal haemoglobin peaks are frequently observed on Bio-Rad cation-exchange HPLC for glycated haemoglobin (HbA1c), particularly in regions with a high prevalence of haemoglobinopathies. These peaks may cause assay failure or clinically relevant bias, but routine data comparing Bio-Rad HPLC with immunoassay methods under such conditions are limited.

Objectives: To determine the frequency of HbA1c reporting failure on Bio-Rad D-10/Variant HPLC in the presence of abnormal peaks, quantify bias versus a Roche Cobas immunoturbidimetric assay, and identify chromatographic predictors of failure and large method differences.

Methods: In this retrospective laboratory study, we analysed 156 unique EDTA blood samples in which Bio-Rad D-10 or Variant chromatograms displayed at least one abnormal haemoglobin peak and a paired HbA1c result was available from a Roche Cobas analyser. Abnormal-peak type, area under the curve (AUC, % of total haemoglobin), retention times of HbA1c and the abnormal peak, and the distance between peaks were recorded. Bio-Rad results were classified as failures when HbA1c was reported as 0% or >20% despite a numeric Cobas result. In samples with numeric results on both methods, we calculated the difference ($\Delta\text{HbA1c} = \text{Bio-Rad} - \text{Cobas}$), the absolute difference ($|\Delta\text{HbA1c}|$), correlation and Bland–Altman limits of agreement. Logistic regression was used to identify predictors of Bio-Rad failure.

Results: Variant-window peaks were the most frequent abnormality (107/156; 68.6%). Based on Cobas HbA1c, 61 patients (39.1%) were normoglycaemic, 37 (23.7%) had prediabetes and 58 (37.2%) had diabetes. Bio-Rad failed in 26/156 chromatograms (16.7%), reporting HbA1c as 0% in 23 and >20% in 3 cases. Failure was more frequent on the Variant than on the D-10 (Variant 14/34, 41.2% vs D-10 12/122, 9.8%). In the 130 analyzable pairs, mean HbA1c was 7.20% by Bio-Rad and 6.62% by Cobas, with a mean bias of 0.58% and limits of agreement from -1.1 to 2.2%. Overall, 28.5% of

paired results differed by ≥ 1.0 percentage point. Abnormal-peak AUC showed a modest positive correlation with $|\Delta\text{HbA1c}|$ ($r=0.19$), whereas greater peak-distance proportion was associated with smaller differences ($r=-0.21$). In multivariable analysis, each 1% increase in AUC increased the odds of Bio-Rad failure (odds ratio 1.12; 95% CI 1.08–1.17), whereas each 0.1-min increase in peak distance reduced the odds (odds ratio 0.34; 95% CI 0.19–0.60).

Conclusions: In samples with abnormal haemoglobin peaks, Bio-Rad HPLC frequently yields non-reportable or positively biased HbA1c values when compared with a Roche Cobas immunoturbidimetric assay. Large variant-window or unknown peaks close to the HbA1c window are the main determinants of failure and bias. Simple chromatogram-based rules using peak area and retention-time separation may help laboratories flag high-risk chromatograms and trigger reflex testing on an alternative HbA1c method.

Keywords: HbA1c; haemoglobin variants; Bio-Rad D-10; Variant; HPLC; Roche Cobas; method comparison; India.

1. Introduction

Glycated haemoglobin (HbA1c) is the cornerstone marker for long-term glycaemic control and is widely used to diagnose and monitor diabetes. International expert groups have recommended the use of HbA1c for diagnosis when assays are traceable to reference methods and meet defined standards of analytical performance [1–3]. Accurate HbA1c measurement is therefore essential for appropriate classification of glycaemic status and therapeutic decision-making.

Cation-exchange high-performance liquid chromatography (HPLC) is a commonly used analytical principle for HbA1c determination. Bio-Rad D-10 and Variant systems are widely implemented, as they combine automated HbA1c reporting with chromatograms that display haemoglobin fractions and variant peaks [4,5]. In regions with a high prevalence of haemoglobinopathies, such as the Indian subcontinent, this dual capability is attractive because HbA1c testing may incidentally reveal previously unrecognised haemoglobin variants.

Haemoglobin variants and other abnormal peaks can, however, interfere with HbA1c measurement. Depending on the variant and assay principle, interference may arise through co-elution with the HbA1c peak, altered red-cell survival or changes in antibody binding. The magnitude and direction of interference vary by method and variant, ranging from negligible effects to large positive or negative biases or complete assay failure [3,6,7]. Reference databases summarise method-specific interferences, but individual laboratories still need local data to guide interpretation of abnormal chromatograms.

Indian studies using Bio-Rad HPLC for haemoglobinopathy screening have reported a high proportion of abnormal chromatograms and characterised the spectrum of variants detected, but have generally not quantified how frequently HbA1c results are misleading, nor which chromatographic features best predict unreliability [8–10]. Case reports describe spuriously high or unreportable HbA1c values on Bio-Rad systems in the presence of specific variants, but these isolated examples do not provide a basis for operational cut-offs.

The present study addresses this gap using routine data from a tertiary-care laboratory in eastern India. We retrospectively identified samples with abnormal haemoglobin peaks on Bio-Rad D-10 or Variant HbA1c programmes that also had paired HbA1c results from a Roche Cobas immunoturbidimetric assay. Our objectives were to describe the spectrum of abnormal chromatograms and the frequency of Bio-Rad reporting failure, quantify agreement and bias between Bio-Rad and Cobas HbA1c across the glycaemic range, and identify simple chromatographic predictors of failure and large method differences.

2. Materials and methods

2.1. Study design and setting

This was a laboratory-based cross-sectional study performed in the clinical biochemistry laboratory of a tertiary-care teaching hospital in eastern India. The laboratory participates in external quality assessment for HbA1c and operates under internal quality-control procedures consistent with international recommendations.

2.2. Case selection

The laboratory information system was searched over a defined multi-year period to identify all HbA1c requests analysed on Bio-Rad D-10 or Variant HPLC in which the chromatogram showed at least one abnormal haemoglobin peak (Variant window, S window, C window, P3, P4, elevated foetal haemoglobin, LA1c/CHb2 or peaks labelled as "unknown"). Only those cases in which a paired HbA1c result was available from a Roche Cobas immunoturbidimetric assay on the same EDTA whole-blood specimen were included. If a patient had multiple eligible samples, only the first chronologically was retained, yielding 156 unique chromatograms for analysis.

2.3. Bio-Rad HPLC methods

Bio-Rad D-10 and Variant analysers use cation-exchange HPLC with dedicated HbA1c programmes. After haemolysis, haemoglobin fractions are separated on an analytical column and detected photometrically. HbA1c is quantified as a percentage of total haemoglobin, aligned to NGSP/IFCC reference methods, and chromatograms display the HbA1c fraction, other haemoglobin fractions and any abnormal peaks [4,5]. The software may assign extreme values (e.g. 0% or >20%) when a large variant peak prevents reliable integration of the HbA1c window; such values are treated as non-reportable in routine practice.

2.4. Roche Cobas HbA1c method

The comparison method was an immunoturbidimetric HbA1c assay on a Roche Cobas analyser. After haemolysis and proteolysis, an antibody specific for the glycosylated N-terminal epitope of the β -chain binds HbA1c and the resulting immune complexes are measured turbidimetrically. The assay is traceable to IFCC reference methods and certified by the NGSP. Published evaluations have shown good precision and minimal interference from common haemoglobin variants for this class of immunoassay, although rare variants and high foetal haemoglobin may still affect results [2,6,7].

2.5. Data extraction and derived variables

From each Bio-Rad chromatogram we recorded the instrument (D-10 or Variant), abnormal-peak type, retention times of the HbA1c and abnormal peaks, and abnormal-peak area expressed as a percentage of total haemoglobin (AUC). Using the instrument-reported percentage of total run time for the abnormal peak, we estimated total run time and calculated the absolute distance between the abnormal and HbA1c peaks in minutes and as a proportion of total run time. Peaks judged by an experienced operator to lie immediately adjacent to the HbA1c window were classified as "adjacent". Bio-Rad HbA1c values were taken directly from the analyser output.

2.6. Outcome measures

Bio-Rad HbA1c results were classified as failures when the analyser reported HbA1c as 0% or >20% and the Cobas assay produced a numeric result for the same specimen. For method-comparison analyses we restricted the dataset to samples with numeric Bio-Rad HbA1c values between 3% and 20% and Cobas HbA1c >0. For each pair we calculated the difference ($\Delta\text{HbA1c} = \text{Bio-Rad} - \text{Cobas}$), the absolute difference ($|\Delta\text{HbA1c}|$) and the mean of the two values. Cobas HbA1c was used to classify glycaemic status as normal (<5.7%), prediabetes (5.7–6.4%) or diabetes ($\geq 6.5\%$).

2.7. Statistical analysis

Continuous variables are reported as mean (standard deviation) or median (interquartile range), and categorical variables as counts and percentages. Agreement between Bio-Rad and Cobas HbA1c was assessed using Pearson correlation and Bland–Altman plots (mean bias and 95% limits of agreement). The proportions of paired results with $|\Delta\text{HbA1c}| \geq 0.5\%$, $\geq 1.0\%$ and $\geq 2.0\%$ were calculated. Because one chromatogram showed an extreme discrepancy ($|\Delta\text{HbA1c}| > 5\%$), a secondary Bland–Altman analysis was performed after excluding this outlier. Associations between absolute method difference and continuous predictors (AUC, peak-distance proportion) were examined using Pearson correlation. Differences across categorical groups (e.g. glycaemic category, instrument) were assessed using t-tests or analysis of variance as appropriate.

To identify independent predictors of Bio-Rad failure, we fitted a multivariable logistic regression model with failure (yes/no) as the dependent variable and abnormal-peak AUC (per 1-percentage-point increase) and peak distance (per 0.1-min increase) as covariates. Odds ratios (ORs) with 95% confidence intervals (CIs) and p-values were reported. A two-sided $p < 0.05$ was considered statistically significant.

2.8. Ethical Considerations

Only anonymised laboratory data generated during routine clinical care were used. No additional samples were collected and no patient-identifying information was retained in the analytical dataset. The institutional ethics committee approved the protocol and waived the requirement for informed consent because of the retrospective, non-interventional design.

3. Results

A total of 156 abnormal Bio-Rad chromatograms with paired Cobas HbA1c results were included. Variant-window peaks accounted for 107/156 (68.6%) of chromatograms, followed by S-window, P4, P3, foetal haemoglobin, C-window, LA1c/CHb2 and unknown peaks. Based on Cobas HbA1c, 61 (39.1%) patients were classified as normoglycaemic, 37 (23.7%) as having prediabetes and 58 (37.2%) as having diabetes.

Bio-Rad HPLC failed to provide a usable HbA1c result (0% or $>20\%$) in 26/156 chromatograms (16.7%). Of these, 23 were reported as 0% and 3 as $>20\%$. Cobas produced numeric HbA1c values in the great majority of these failures. Failure rates differed by instrument: the D-10 failed in 12/122 chromatograms (9.8%), whereas the Variant failed in 14/34 (41.2%). Failures were concentrated in chromatograms with very large Variant-window or unknown peaks; no failures were observed in chromatograms with isolated S-window, P3, P4, foetal haemoglobin or C-window peaks.

In the 130 analysable pairs with numeric results on both methods, mean HbA1c was 7.20% (standard deviation 1.80) by Bio-Rad and 6.62% (standard deviation 1.73) by Cobas. The correlation between methods was strong ($r = 0.885$, $p < 0.001$). However, Bio-Rad values tended to be higher, with a mean difference (Bio-Rad – Cobas) of 0.58% and 95% limits of agreement from -1.1 to 2.2%. Overall, 75.4% of paired results differed by at least 0.5 percentage points, 28.5% by $\geq 1.0\%$ and 3.8% by $\geq 2.0\%$.

One chromatogram showed an extreme discrepancy with $|\Delta\text{HbA1c}|$ greater than 5 percentage points. After excluding this outlier ($n = 129$), the mean bias remained positive at 0.54% and the limits of agreement narrowed to -0.92 to 2.00%. Even in this trimmed dataset, approximately 27.9% of paired results still differed from Cobas by at least 1.0 percentage point. The scatter plot and Bland–Altman plot are shown in Figures 1 and 2.

Across analysable pairs, abnormal-peak AUC was modestly but significantly correlated with absolute method difference $|\Delta\text{HbA1c}|$ ($r = 0.19$, $p = 0.033$). Peak-distance proportion showed a weak negative correlation with $|\Delta\text{HbA1c}|$ ($r = -0.21$, $p = 0.018$), indicating that peaks eluting closer to the HbA1c fraction were more likely to distort the result. Mean absolute differences did not differ significantly across Cobas-defined glycaemic categories (normal, prediabetes, diabetes).

In multivariable logistic regression including abnormal-peak AUC and peak distance (per 0.1-min increase), both variables independently predicted Bio-Rad failure. Each 1-percentage-point increase in AUC increased the odds of failure (odds ratio 1.12; 95% CI 1.08–1.17; $p=3.467\text{e-}09$), whereas greater separation between the abnormal peak and the HbA1c fraction reduced the odds (odds ratio 0.34 per 0.1-min increase; 95% CI 0.19–0.60; $p=2.085\text{e-}04$). These findings support the use of peak size and distance as simple quantitative markers for identifying chromatograms at high risk of Bio-Rad HbA1c failure.

4. Discussion

In this cohort of 156 abnormal Bio-Rad HbA1c chromatograms with paired Cobas results, we observed that assay failure and clinically relevant disagreement were common. Approximately one in six chromatograms yielded a non-usable Bio-Rad HbA1c value despite a numeric Cobas result, and more than a quarter of analysable pairs differed from Cobas by at least 1.0 percentage point. These discrepancies were strongly linked to simple chromatographic features: abnormal-peak size and proximity to the HbA1c window.

Our findings are consistent with evidence that haemoglobin variants and related abnormalities can interfere with HbA1c measurement in a method-dependent manner. Reviews and NGSP resources emphasise that certain variants and high foetal haemoglobin may cause substantial bias or assay failure on some platforms while leaving others unaffected [2,3,6,7]. Case reports and small series have described spuriously high or unreportable HbA1c values on Bio-Rad systems in association with variants such as Hb D Punjab, Hb Q India and Hb Hope, but have not provided quantitative decision thresholds [8–10]. Our study extends this literature by showing, in routine practice, that large variant-window or unknown peaks are the main drivers of Bio-Rad failure and positive bias when compared with an immunoturbidimetric assay.

Two observations are particularly relevant for laboratory practice. First, abnormal-peak AUC emerged as a powerful predictor of failure, with odds increasing by about 12% for each 1-percentage-point increment in AUC. In practical terms, chromatograms in which the abnormal peak occupied more than half of the total haemoglobin were rarely associated with a trustworthy Bio-Rad HbA1c result. Second, greater separation between the abnormal peak and the HbA1c fraction was protective, suggesting that co-elution or close elution is a key mechanism of interference. These parameters are readily available from the chromatogram and could be incorporated into simple rules for reflex testing.

From a clinical perspective, the predominantly positive bias observed for Bio-Rad relative to Cobas is important. Overestimation of HbA1c may lead to inappropriate intensification of glucose-lowering therapy or misclassification of patients as diabetic or poorly controlled. This risk is greatest when HbA1c is used for diagnosis or when results lie near clinical decision thresholds. Our data therefore support a cautious, algorithm-based approach in which chromatograms with large, closely eluting variant peaks trigger automatic review and, where available, repeat HbA1c measurement using an alternative NGSP-certified method that has been evaluated for local variant patterns.

The present study has several limitations. It reflects the experience of a single tertiary-care laboratory and may not be generalisable to settings with different haemoglobinopathy spectra or analytical platforms. We did not perform systematic electrophoresis or genetic testing, so chromatographic patterns could not be definitively linked to specific variants. The Cobas immunoturbidimetric assay, although well standardised, is not a perfect reference and may itself be affected by rare variants or abnormal red-cell turnover. Finally, some subgroup analyses were based on relatively small numbers of chromatograms, limiting statistical power.

Despite these limitations, the study provides pragmatic information for laboratories that rely on Bio-Rad HPLC for HbA1c measurement in high-prevalence haemoglobinopathy regions. It shows that a combination of visual review and quantitative thresholds for abnormal-peak area and distance can be used to flag high-risk chromatograms. Prospective validation of area- and distance-based

rules, coupled with systematic variant identification and clinical follow-up, would be a valuable next step.

5. Conclusions

Abnormal haemoglobin peaks on Bio-Rad D-10 and Variant HbA1c chromatograms are common in our practice and frequently compromise the reliability of HbA1c reporting. Large variant-window or unknown peaks, especially when located close to the HbA1c window, are associated with both outright assay failure and substantial positive bias relative to a Roche Cobas immunoturbidimetric assay. Incorporating simple chromatogram-based rules based on abnormal-peak area and retention-time separation, and reflex testing of high-risk samples using an alternative HbA1c method, may improve the safety and interpretability of HbA1c results in patients with haemoglobin variants.

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Author contributions

Concept and study design: RS, TG. Data extraction and analysis. TG, AR. Drafting of the manuscript: All. Critical revision of the manuscript: All. All authors approved the final version.

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Conflict of interest

The authors declare no conflicts of interest related to this work.

Figures

Figure 1. Scatter plot of HbA1c measured by Bio-Rad HPLC versus Roche Cobas immunoturbidimetric assay in analyzable samples.

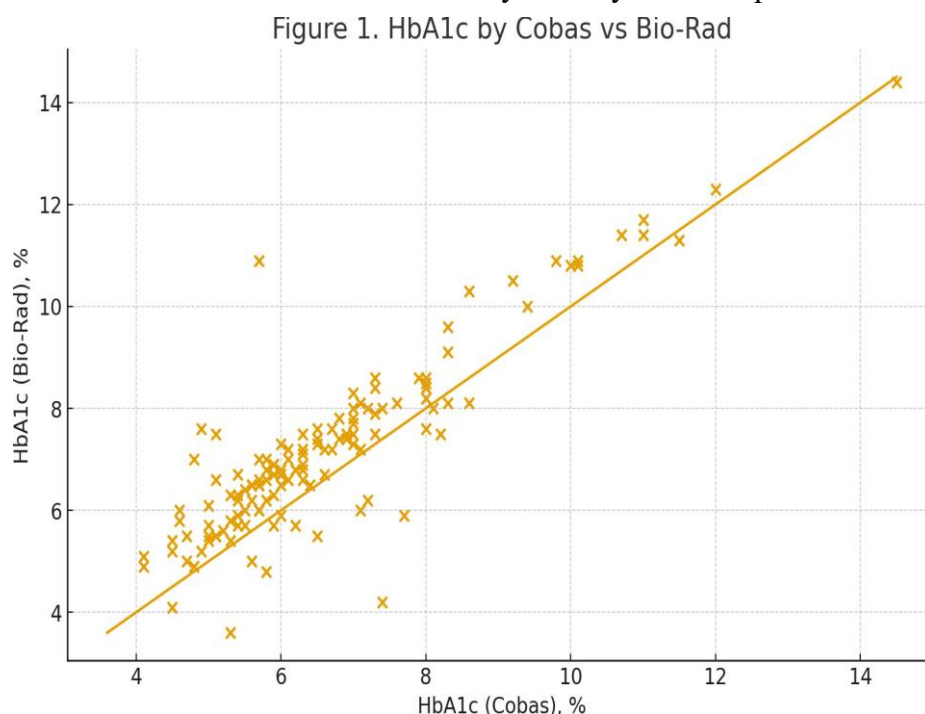


Figure 2. Bland–Altman plot of the difference between Bio-Rad and Cobas HbA1c versus mean HbA1c after exclusion of one extreme outlier ($|\Delta\text{HbA1c}| > 5$ percentage points).

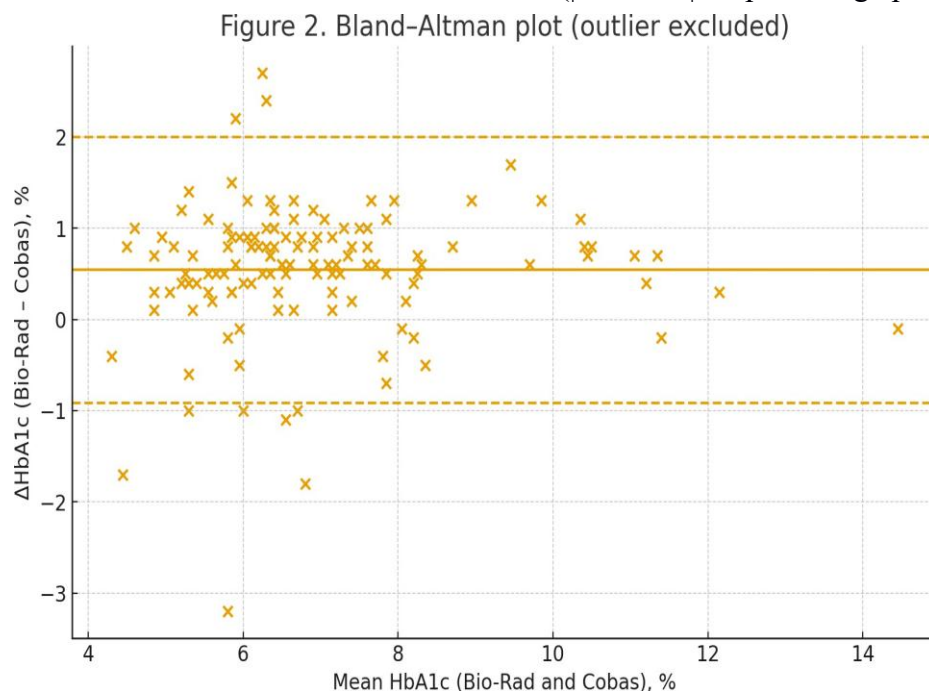
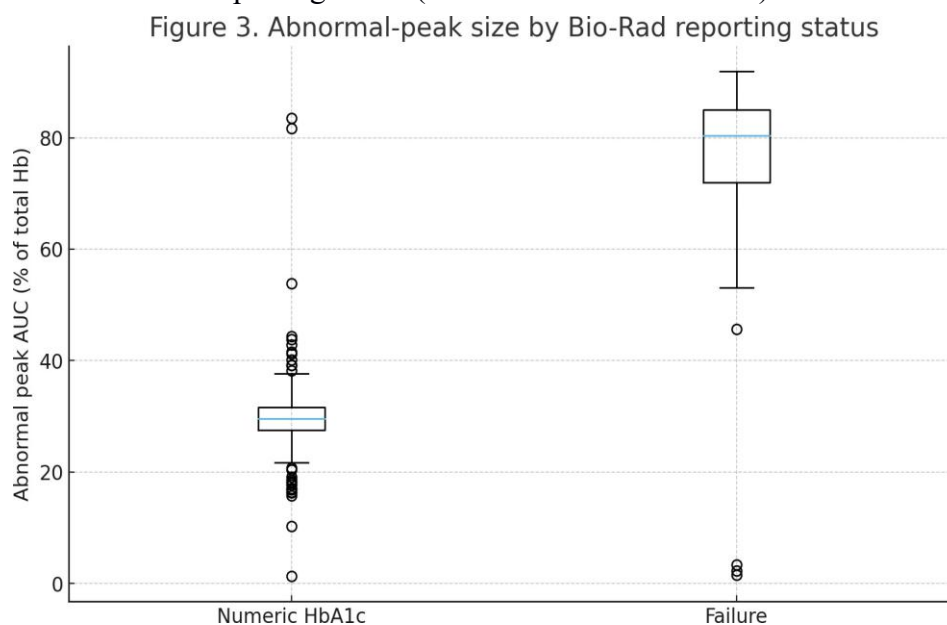


Figure 3. Boxplot of abnormal-peak area (percentage of total haemoglobin) stratified by Bio-Rad reporting status (numeric HbA1c vs failure).



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