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FLUORESCENCE-BASED HPLC QUANTIFICATION OF NEOPTERIN AND TOTAL NEOPTERIN IN HUMAN PLASMA: PRACTICAL ANALYTICAL CONSIDERATIONS AND CLINICAL APPLICATION

Siddarth Raajasekar[1]*

[1] Department of Biotechnology, KIT-Kalaingar Karunanidhi Institute of Technology, Coimbatore, Tamil Nadu, India
*Corresponding author: siddarthraajasekarbiotech@kitcbe.ac.in

ABSTRACT
This work describes the use of fluorescence-based HPLC to quantify neopterin and total neopterin in human plasma. Blood collection, plasma preparation, acetonitrile protein precipitation, triiodide-induced selective oxidation of 7,8-dihydroneopterin to neopterin, isocratic chromatographic separation on an amino-bonded silica stationary phase, and fluorescence detection are the analytical steps. The clinical cohort data discussed herein were previously reported by Raajasekar et al. (2025) and are reinterpreted here exclusively from an analytical and preparative-biotechnology perspective. The original clinical study did not report formal analytical validation parameters such as recovery, precision, linearity, limit of detection, limit of quantification, and matrix effect assessment. Future validation work should address these parameters. The manuscript provides systematic steps to address analytical issues such as poor peak shape, variable triiodide oxidation, fluorescence quenching, and baseline instability using evidence-based methods. Plasma and total neopterin levels were significantly higher in stroke patients compared to age-matched controls in 61 carotid endarterectomy patients (Raajasekar et al., 2025) (11.3 vs. 7.7 nM, $p = 0.0372$ and 46.37 vs. 19.29 nM, $p = 0.0001$). This manuscript analyses the dataset from an analytical methodology perspective, focusing on sample preparation, chromatographic workflow, triiodide oxidation, and quantification of plasma and total neopterin.

KEYWORDS: neopterin; 7,8-dihydroneopterin; total neopterin; HPLC; fluorescence detection; plasma sample preparation; triiodide oxidation; amino-bonded silica; preparative biochemistry; analytical biotechnology

1. INTRODUCTION

Neopterin (D-erythro-1′,2′,3′-trihydroxypropylpterin) is a pteridine derivative synthesised and released by human monocytes and macrophages upon stimulation with interferon-γ (IFN-γ), making it a well-established biomarker of cellular immune activation in cardiovascular disease, infectious diseases, autoimmune disorders, and malignancies (Pacileo et al., 2007). The biosynthetic pathway involves guanosine triphosphate cyclohydrolase I-mediated conversion of guanosine triphosphate to 7,8-dihydroneopterin triphosphate, which is subsequently dephosphorylated to yield 7,8-dihydroneopterin. Partial oxidation of 7,8-dihydroneopterin by reactive oxygen species in the inflammatory microenvironment generates neopterin, thereby establishing a direct biochemical link among macrophage activation, oxidative stress, and pteridine metabolism (Gieseg et al., 2010; Baxter-Parker et al., 2020).
The concept of total neopterin — defined as the sum of neopterin and 7,8-dihydroneopterin after chemical oxidation — has emerged as a more comprehensive indicator of immune activation, independent of the local oxidative environment (Baxter-Parker et al., 2021). 7,8-Dihydroneopterin functions as a potent antioxidant, scavenging superoxide radicals and reducing the uptake of oxidized low-density lipoprotein by macrophages (Gieseg & Esterbauer, 1994; Gieseg et al., 2010). Measurement of neopterin alone may underestimate the degree of macrophage activation when oxidative stress is low, as 7,8-dihydroneopterin may predominate under such conditions. Simultaneous measurement of both pteridines, or measurement of total neopterin, therefore provides a more complete assessment of immune activation status (Raajasekar et al., 2025).
Elevated plasma neopterin concentrations have been reported in patients with ischaemic stroke (Anwaar et al., 1999; Xie et al., 2019; Meng & Cao, 2021; Alme et al., 2021), and neopterin has been identified within atherosclerotic plaques (Janmale et al., 2015). However, relatively few studies have reported total neopterin in cardiovascular disease

cohorts, and the analytical workflow required to measure both neopterin and total neopterin in plasma presents specific practical challenges that are not always addressed in detail in the clinical literature.

Fluorescence-based HPLC on amino-bonded silica columns provides a selective and sensitive approach for pteridine separation from biological matrices (Baxter-Parker et al., 2019a; Baxter-Parker et al., 2019b; Lindsay et al., 2019). The method relies on the intrinsic fluorescence of the pteridine ring system and requires protein precipitation prior to injection, as well as a separate triiodide oxidation step for total neopterin measurement. Each of these steps introduces potential sources of analytical variability that must be understood and managed in practice.

The aim of this manuscript is to provide a practical, step-by-step description of the analytical workflow for plasma neopterin and total neopterin quantification, with particular attention to common sources of error and evidence-based troubleshooting strategies. As a clinical application case study, the methodology is illustrated using data from a previously published cohort of 61 carotid endarterectomy patients and 61 age-matched controls (Raajasekar et al., 2025). The clinical cohort data used as an analytical application case study were previously reported by Raajasekar et al. (2025). The present manuscript reinterprets the previously published dataset from an analytical-methodology perspective and does not report newly generated clinical cohort data.

2. MATERIALS AND METHODS

2.1 Reagents and Standards

Neopterin standard was obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC grade), potassium iodide, iodine, and ascorbic acid were obtained from commercial suppliers. All aqueous solutions were prepared using ultrapure water (≥18 MΩ·cm). Ammonium acetate buffer (0.02 M, pH 6.9) was used as the mobile phase.

2.2 Blood Collection and Plasma Preparation

Venous blood was collected into EDTA-containing tubes (K_2EDTA, 3 mL). Tubes were inverted gently five to eight times to ensure anticoagulant mixing, then centrifuged at 1,500 × g for 10 minutes at 4°C within 30 minutes of collection. Plasma was carefully aspirated with a transfer pipette, avoiding the buffy coat, and stored in 1.5 mL microcentrifuge tubes at −80°C until analysis. The blood collection and plasma processing procedures for the clinical cohort were as described by Raajasekar et al. (2025).

2.3 Protein Precipitation

Plasma samples were thawed on ice. Acetonitrile was added to each plasma sample at a 1:1 (v/v) ratio, and the samples were vortexed for 30 seconds. Samples were centrifuged at 12,000 × g for 10 minutes at 4°C. The clarified supernatant was transferred to a fresh tube and used directly for HPLC injection (Path A, neopterin) or subjected to triiodide oxidation (Path B, total neopterin).

2.4 Triiodide Oxidation for Total Neopterin

Triiodide reagent was prepared by dissolving iodine (3.2 mM) and potassium iodide (6.4 mM) in ultrapure water. The clarified supernatant was mixed with triiodide reagent at a 1:1 (v/v) ratio and incubated at room temperature in the dark for 30 minutes. After oxidation, excess iodine was quenched by the addition of ascorbic acid solution (0.1 M) in a volume sufficient to decolourise the solution. The quenched sample was centrifuged briefly to remove any precipitate before HPLC injection.

2.5 HPLC-FLD Analysis

Chromatographic separation was performed on an amino-bonded silica column (e.g., Supelcosil LC-NH_2, 250 × 4.6 mm, 5 µm particle size) using isocratic elution with 0.02 M ammonium acetate buffer (pH 6.9) at a flow rate of 1.0 mL/min. The column temperature was maintained at 30°C. Fluorescence detection was performed at excitation 353 nm and emission 438 nm. The injection volume was 50 µL. Calibration was performed using aqueous neopterin standards prepared in mobile phase.

3. Analytical Workflow Development

The analytical workflow for quantifying plasma neopterin and total neopterin involves two parallel sample-preparation paths from a single plasma aliquot. Path A provides direct measurement of neopterin, while Path B incorporates a triiodide oxidation step to convert all 7,8-dihydroneopterin to neopterin, enabling measurement of total neopterin. The 7,8-dihydroneopterin concentration is then calculated as the arithmetic difference between total neopterin and neopterin. The analytical workflow for parallel neopterin and total neopterin quantification is shown in Figure 1.

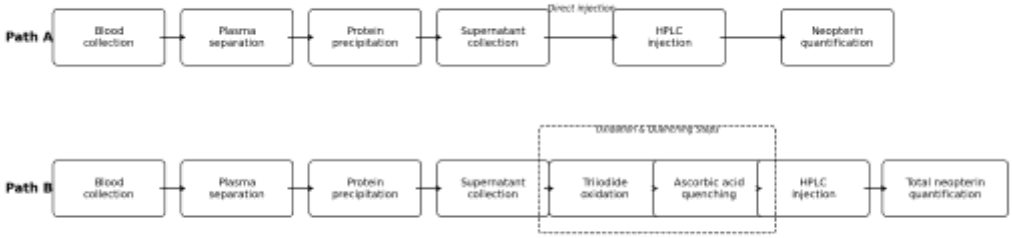

Figure 1. Analytical workflow for plasma neopterin and total neopterin quantification. Path A (upper) shows the direct injection pathway for neopterin measurement. Path B (lower) incorporates triiodide oxidation and ascorbic acid quenching for total neopterin measurement. The 7,8-dihydroneopterin concentration is calculated as the difference between total neopterin and neopterin values.

The complete sample preparation workflow is summarised in Table 1.

Table 1. Sample preparation workflow for plasma neopterin and total neopterin quantification. 7,8-DHNP = 7,8-dihydroneopterin. All centrifugation steps at 4°C. Protect samples from light during and after oxidation.

Step	Path A (Neopterin)	Path B (Total Neopterin)
1	Collect venous blood into a K_2EDTA tube	Collect venous blood into a K_2EDTA tube
2	Centrifuge at 1,500 × g, 10 min, 4°C	Centrifuge at 1,500 × g, 10 min, 4°C
3	Aspirate plasma; store at −80°C	Aspirate plasma; store at −80°C
4	Thaw on ice; add acetonitrile 1:1 (v/v)	Thaw on ice; add acetonitrile 1:1 (v/v)
5	Vortex 30 s; centrifuge 12,000 × g, 10 min	Vortex 30 s; centrifuge 12,000 × g, 10 min
6	Collect supernatant	Collect supernatant
7	Inject directly onto the HPLC column	Add triiodide reagent 1:1 (v/v); incubate 30 min, dark
8	Quantify the neopterin peak	Quench with ascorbic acid; centrifuge briefly
9	—	Inject onto HPLC column; quantify total neopterin
10	—	Calculate 7,8-DHNP = Total neopterin − Neopterin

4. Sample Preparation Considerations

4.1 Blood Collection Timing

The interval between blood collection and plasma separation should be minimised. Prolonged storage of whole blood prior to centrifugation may result in ex vivo oxidation of 7,8-dihydroneopterin to neopterin, artificially elevating neopterin and reducing 7,8-dihydroneopterin values. Centrifugation within 30 minutes of collection is recommended.

4.2 Protein Precipitation

Acetonitrile precipitation at a 1:1 (v/v) ratio effectively removes plasma proteins while maintaining adequate pteridine recovery. The resulting supernatant should be clear; turbidity indicates incomplete precipitation and may cause column fouling. If turbidity persists after centrifugation, a second centrifugation step or filtration through a 0.22 µm membrane filter may be applied. It should be noted that formal recovery experiments were not performed in the original clinical study (Raajasekar et al., 2025), and dedicated recovery assessment should be included in future validation work.

4.3 Triiodide Oxidation

The triiodide oxidation step converts 7,8-dihydroneopterin to neopterin quantitatively under appropriate conditions. Critical variables include the triiodide concentration, incubation time, and pH. Incomplete oxidation leads to

underestimation of total neopterin, while over-oxidation or prolonged exposure to excess iodine may degrade neopterin. Ascorbic acid quenching must be performed promptly and in sufficient quantity to fully decolourise the solution before injection. Residual iodine in the injection solution may cause column damage and detector interference.

4.4 Sample Storage

Plasma samples should be stored at −80°C in aliquots to avoid repeated freeze-thaw cycles. Freeze-thaw stability data were not reported in the original clinical study (Raajasekar et al., 2025), and systematic freeze-thaw stability experiments should be included in future validation work.

5. HPLC Method Description

5.1 Stationary Phase

Amino-bonded silica columns (NH_2) provide appropriate selectivity for pteridine separation from biological matrix components under aqueous isocratic conditions. The amino functional groups interact with the pteridine ring system through a combination of polar and weak ionic interactions, enabling resolution of neopterin from co-eluting matrix peaks.

5.2 Mobile Phase and Isocratic Conditions

Isocratic elution with 0.02 M ammonium acetate buffer (pH 6.9) at 1.0 mL/min provides stable baseline conditions and reproducible retention times for neopterin. The low ionic strength and near-neutral pH are compatible with the amino-bonded stationary phase and minimise background fluorescence. Mobile phase should be filtered through a 0.22 µm membrane and degassed prior to use.

5.3 Fluorescence Detection

Neopterin exhibits strong intrinsic fluorescence with excitation at 353 nm and emission at 438 nm, providing high selectivity relative to most plasma matrix components. Detector gain settings should be optimised for the expected concentration range in the samples under analysis. Detector lamp warm-up time (typically 15–30 minutes) should be observed before sample injection to ensure a stable baseline fluorescence.

5.4 Retention Time and Calibration

Under the described isocratic conditions, neopterin elutes at approximately 8.5–9.0 minutes on a 250 mm amino-bonded column at 1.0 mL/min. Calibration is performed using aqueous neopterin standards prepared in the mobile phase. A minimum of five calibration points spanning the expected sample concentration range is recommended. Calibration standards should be prepared fresh and analysed at the beginning and end of each analytical run. Representative chromatograms illustrating direct neopterin measurement and post-oxidation total neopterin measurement are shown in Figure 3.

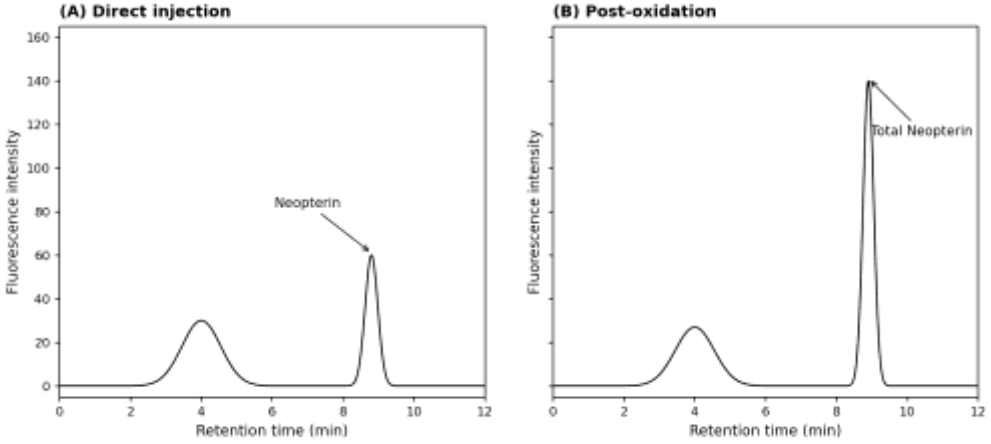

Figure 3. Representative HPLC-FLD chromatograms illustrating the two parallel analytical pathways. (A) Direct injection: the neopterin peak elutes at approximately 8.5–9.0 minutes. A smaller early-eluting peak represents matrix components. (B) Post-oxidation injection: after triiodide oxidation of 7,8-dihydroneopterin to neopterin, the total neopterin peak is larger, reflecting the sum of both pteridines. Fluorescence intensity (arbitrary units) is shown on the y-axis; retention time (min) on the x-axis.

The final HPLC-FLD method conditions are summarised in Table 2.

Table 2. HPLC-FLD method conditions for quantification of plasma neopterin and total neopterin.

Parameter	Condition
Column	Amino-bonded silica (NH_2), 250 × 4.6 mm, 5 µm

Mobile phase	0.02 M ammonium acetate, pH 6.9
Elution mode	Isocratic
Flow rate	1.0 mL/min
Column temperature	30°C
Injection volume	50 µL
Detection	Fluorescence (Ex 353 nm / Em 438 nm)
Approx. Neopterin retention time	8.5–9.0 min
Calibration	Aqueous neopterin standards in the mobile phase
Run time	~12 min

6. Analytical Considerations and Troubleshooting

Fluorescence-based HPLC quantification of plasma pteridines is susceptible to a range of analytical problems that can affect peak shape, signal intensity, and run-to-run reproducibility. A practical troubleshooting workflow for the most common analytical problems is shown in Figure 2.

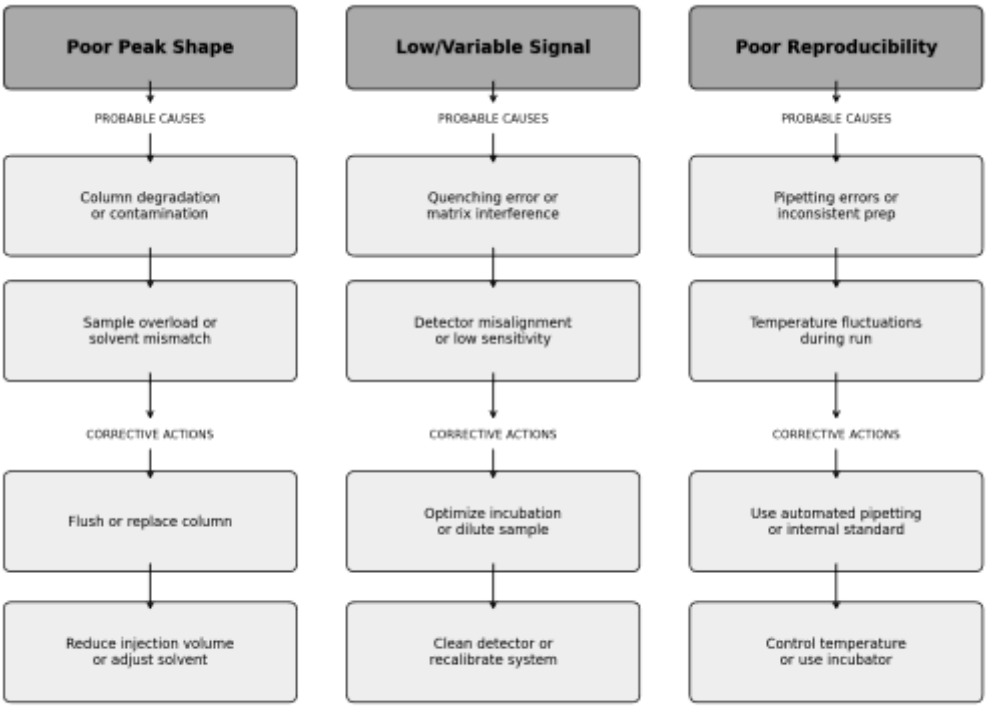

Figure 2. Practical troubleshooting workflow for the most common analytical problems encountered in HPLC-FLD quantification of plasma pteridines. Three primary problem categories are shown: poor peak shape, low or variable signal, and poor reproducibility. For each category, probable causes and recommended corrective actions are listed.

Common analytical problems, likely causes, and corrective actions are summarised in Table 3.

Table 3. Common analytical pitfalls and troubleshooting recommendations for HPLC-FLD plasma pteridine quantification.

Problem	Probable Cause(s)	Corrective Action
Poor peak shape (tailing, broadening)	Column degradation or contamination; sample overload; solvent mismatch	Flush or replace column; reduce injection volume; prepare samples in mobile phase

Low or variable fluorescence signal	Quenching error (excess iodine); matrix interference; detector misalignment	Verify ascorbic acid quenching; optimise incubation; clean or recalibrate detector
Elevated baseline or drift	Contaminated mobile phase; column bleed; insufficient warm-up	Prepare fresh mobile phase; filter and degas; allow ≥15 min lamp warm-up
Poor run-to-run reproducibility	Pipetting errors, inconsistent incubation times, and temperature fluctuations	Use calibrated pipettes; standardise incubation timing; control column temperature
Absent or very small neopterin peak	Insufficient sample; incorrect detector settings; failed protein precipitation	Verify sample preparation; check detector wavelength settings
Incomplete triiodide oxidation	Insufficient triiodide concentration; short incubation; incorrect pH	Prepare fresh triiodide reagent; extend incubation to 30 min; verify buffer pH

7. Clinical Application Case Study

The clinical cohort data used in an analytical application case study were previously reported by Raajasekar et al. (2025) and are reinterpreted here from an analytical methodology perspective. The present manuscript does not report newly generated clinical cohort data.

Plasma neopterin and total neopterin were measured by HPLC-FLD in 61 carotid endarterectomy patients presenting with stroke and 61 age-matched healthy controls (Raajasekar et al., 2025). Blood was collected into EDTA tubes, and plasma was prepared as described in Section 2 above. Neopterin was measured by direct injection (Path A), and total neopterin was measured after triiodide oxidation (Path B). 7,8-Dihydroneopterin was calculated as the difference between total neopterin and neopterin.

Neopterin was significantly elevated in stroke patients compared with age-matched controls (11.3 nM vs. 7.7 nM; $p = 0.0372$). Total neopterin showed a larger and more significant difference (46.37 nM vs. 19.29 nM; $p = 0.0001$). Calculated 7,8-dihydroneopterin was also significantly higher in stroke patients (34.24 nM vs. 12.9 nM; $p < 0.001$). Plasma IL-1β showed no significant difference between stroke patients and controls (Raajasekar et al., 2025). These findings demonstrate that total neopterin, which captures both the oxidised and reduced pteridine species, provides a more sensitive indicator of macrophage activation than neopterin alone.

The clinical application of this analytical workflow is summarised graphically in Figure 4.

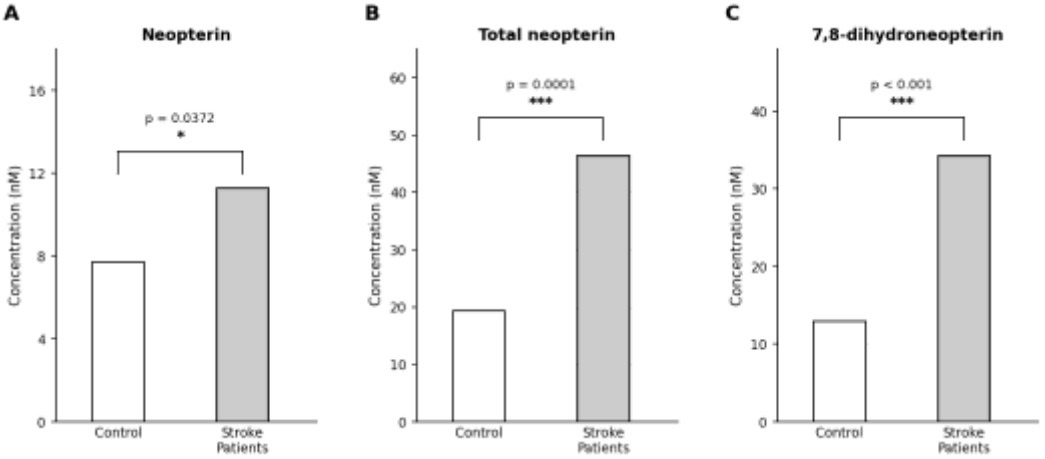

Figure 4. Clinical application of plasma pteridine measurement in stroke/carotid endarterectomy patients and age-matched controls. Data are derived from the previously published cohort reported by Raajasekar et al. (2025). (A) Neopterin: controls 7.7 nM versus stroke patients 11.3 nM, p = 0.0372. (B) Total neopterin: controls 19.29 nM versus stroke patients 46.37 nM, p = 0.0001. (C) Calculated 7,8-dihydroneopterin: controls 12.9 nM versus stroke patients 34.24 nM, p < 0.001. The figure is intended as an analytical application summary and does not represent newly generated clinical data.

The clinical application summary is presented in Table 4.

Table 4. Clinical application summary: plasma pteridine levels in stroke/carotid endarterectomy patients versus age-matched controls (Raajasekar et al., 2025). Data are mean values only. Dispersion values (SD or SEM) were not available for independent verification and are therefore not reported. Source: Raajasekar et al. (2025), https://doi.org/10.1515/pteridines-2025-0055.

Analyte	Controls (n = 61) Mean (nM)	Stroke Patients (n = 61) Mean (nM)	p-value
Neopterin	7.7	11.3	0.0372
Total neopterin	19.29	46.37	0.0001
7,8-Dihydroneopterin (calculated)	12.9	34.24	< 0.001

8. DISCUSSION

The results from the clinical application case study (Raajasekar et al., 2025) demonstrate that the dual-pathway HPLC-FLD workflow described here can detect biologically meaningful differences in plasma pteridine concentrations between stroke patients and healthy controls. The substantially greater elevation in total neopterin than in neopterin alone (approximately 2.4-fold versus 1.5-fold increases) underscores the importance of measuring both pteridine species, as previously discussed in the context of urinary pteridine monitoring in arthroplasty patients (Baxter-Parker et al., 2021) and exercise-associated oxidative stress (Baxter-Parker et al., 2019a).

The absence of a significant difference in IL-1β between stroke patients and controls (Raajasekar et al., 2025) is notable and suggests that macrophage activation, as reflected by pteridine elevations, may precede or occur independently of the IL-1β-mediated inflammatory cascade in this patient group. This observation is consistent with the known biology of 7,8-dihydroneopterin as a macrophage-derived antioxidant whose release is driven primarily by IFN-γ rather than IL-1β signalling (Gieseg et al., 2010; Baxter-Parker et al., 2020).

From an analytical perspective, the magnitude of the total neopterin elevation in stroke patients (mean 46.37 nM) relative to controls (mean 19.29 nM) is well within the quantifiable range of the HPLC-FLD method under the conditions described, indicating that the method provides adequate sensitivity for clinical application in this population. The calculated 7,8-dihydroneopterin values (34.24 nM in patients vs. 12.9 nM in controls) are derived from the arithmetic difference between total neopterin and neopterin and therefore carry the combined analytical uncertainty of both measurements; this should be considered when interpreting 7,8-dihydroneopterin data.

Several methodological considerations merit attention. First, the amino-bonded silica column is susceptible to gradual degradation during repeated use with biological matrix injections; regular quality-control injections and column replacement schedules are recommended. Second, the triiodide oxidation step introduces additional variability, and preparing fresh reagent before each analytical run is advisable. Third, calibration standards prepared in an aqueous mobile phase do not account for potential matrix effects on pteridine recovery or detector response; future validation work should include matrix-matched calibration or standard-addition approaches.

It is important to note that formal analytical validation — including assessment of linearity, accuracy, precision (intra- and inter-day), recovery, limit of detection, limit of quantification, matrix effects, and freeze-thaw stability — was not performed in the original clinical study (Raajasekar et al., 2025). The present manuscript describes a practical analytical methodology and does not constitute a fully validated method. Future work should address these validation parameters in accordance with applicable guidelines (e.g., ICH Q2(R1) or equivalent).

9. Limitations and Future Validation Requirements

The principal limitations of the present work are as follows:

Formal validation not performed. Analytical validation parameters, including recovery, precision (intra- and inter-day), linearity, limit of detection, limit of quantification, matrix effects, and freeze-thaw stability, were not reported in the original clinical study (Raajasekar et al., 2025) and are not presented here. Future dedicated validation experiments should address all parameters in accordance with ICH Q2(R1) or equivalent guidelines.

Matrix-matched calibration. Calibration was performed using aqueous standards. Matrix-matched calibration or standard-addition approaches should be evaluated to determine whether plasma matrix effects significantly affect quantitative accuracy.

Single-laboratory data. The clinical application data are derived from a single published study (Raajasekar et al., 2025). Multi-laboratory reproducibility and interlaboratory comparison data are not available.

Freeze-thaw stability. The effect of repeated freeze-thaw cycles on plasma pteridine concentrations has not been systematically evaluated in this analytical context. Future studies should include controlled freeze-thaw stability experiments.

Column lot variability. Amino-bonded silica columns from different manufacturers or column lots may show differences in selectivity and retention. Method transfer to a new column should include a verification step using reference standards and quality control samples.
Dispersion data. Only mean values are reported in Table 4, as SD and SEM values were not independently verified against the source material. Readers requiring dispersion data should consult the original publication (Raajasekar et al., 2025).

10. CONCLUSIONS

This manuscript describes a practical HPLC-FLD workflow for the simultaneous quantification of neopterin and total neopterin in human plasma. The dual-pathway approach — direct injection for neopterin and triiodide oxidation for total neopterin — enables calculation of 7,8-dihydroneopterin as the difference between the two measurements, providing a more complete assessment of macrophage activation status than neopterin measurement alone. The manuscript identifies the principal sources of analytical variability at each stage of the workflow and provides evidence-based troubleshooting guidance.
As a clinical application case study, the method is illustrated using previously published data from 61 stroke/carotid endarterectomy patients and 61 age-matched controls (Raajasekar et al., 2025), in which total neopterin showed the greatest and most statistically significant elevation between groups. These findings are consistent with the analytical rationale for measuring total neopterin rather than neopterin alone in inflammatory disease contexts.
Formal analytical validation — including recovery, precision, linearity, LOD, LOQ, matrix effects, and freeze-thaw stability — was not performed in the original clinical study and represents an important area for future work. Laboratories implementing this method are encouraged to conduct appropriate validation experiments before clinical application.

Ethics Statement

The clinical cohort data discussed in Section 7 and presented in Table 4 and Figure 4 were previously reported by Raajasekar et al. (2025). Ethical approval and informed consent for that study were obtained as described in the original publication. No new human participants were recruited for the present manuscript.

Informed Consent Statement

Informed consent was obtained from all participants in the original clinical cohort study (Raajasekar et al., 2025). No new participant data are presented in this manuscript.

Data Availability Statement

The clinical data presented in Table 4 and Figure 4 are derived from Raajasekar et al. (2025) and are available in the original publication at https://doi.org/10.1515/pteridines-2025-0055. No new primary data are generated in this manuscript.

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Conflict of Interest Statement

The authors declare no conflicts of interest.

Author Contributions

S.R.: conceptualisation, analytical workflow development, manuscript preparation, and revision.

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Declaration of Generative AI Use

During manuscript preparation, generative AI-assisted tools were used only for language polishing, structural organisation, formatting support, and journal-compliance checking. The authors verified the originality, accuracy, scientific content, references, and DOI information. The authors take full responsibility for the integrity of the

manuscript, including the accuracy of all citations, references, figures, tables, and conclusions. No generative AI tool was used to generate, analyse, or interpret experimental data.

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