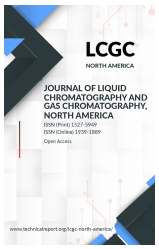

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RESEARCH ARTICLE

Paired Assay Balance and Green Derivative UV Quantification of Brinzolamide–Timolol Ophthalmic Products

Peter W. Carr[1*], **Malcolm Nicoll**[1] **and Robert Freeman**[1]

[1]*Professor of Chemistry, University of Minnesota*
[2]*Department of Chemistry, Faculty of Science, Sakarya Applied Sciences University, Sakarya, Türkiye*
[*]*Corresponding author*

Corresponding author:
petecarr@umn.edu

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Abstract

Fixed dose ophthalmic pharmaceuticals made up of brinzolamide (BRZ) and timolol maleate (TML) must be characterized through an analysis which enables quantitative determination of both substances in a single mixture without depending on the use of solvent for routine product quality control. In this case, the present paper will assess if the first order derivative UV-spectrophotometric method could provide adequate basis for making decisions concerning the quality control of BRZ-TML products taking into consideration their calibration margin, recovery, balance of formulation, and environmental cost. Analytical data included derivative wavelengths 248.80 nm and 297.60 nm for BRZ and TML respectively; calibration interval range 4-24 and 5-25 µg mL^{-1}; detection and quantification limits; 6 triplicate recovery series; assay values of three commercially available formulations and one in situ gel; as well as greenness values from AGREE, eco-scale, and penalty point calculations. Linearity coefficients of R^2=0.9998 and R^2=0.9999 were determined in the case of BRZ and TML respectively. Quantification limits of 0.91 µg mL^{-1} (BRZ) and 2.99 µg mL^{-1} (TML) were found, corresponding to a sensitivity ratio of 4.40 and 1.67 against the first calibration point. Recoveries varied between 97.43-103.22% (BRZ) and 101.73-103.34% (TML), where all relative standard deviations were under 2%. Assay values of the test products ranged between 102.63-105.39% for BRZ and 102.52-105.68% for TML. An in situ gel preparation gave the most balanced paired assay with only 0.03 percentage point difference between BRZ and TML. The derivative UV method further yielded AGREE score 0.77, eco-scale score 88, and 12 penalty points reflecting its low environmental impact as compared to its competitor, the chromatographic method.

Keywords: brinzolamide; timolol maleate; derivative UV spectrophotometry; paired assay; green analytical chemistry; ophthalmic suspension; analytical validation

1. Introduction

The main advantage of topical therapy for glaucoma and ocular hypertension is fixed-dose combination products that simplify dosing while ensuring complementary pathways of intraocular-pressure reduction. Thus, brinzolamide (BRZ) is a carbonic anhydrase inhibitor that reduces aqueous humor production, and timolol maleate (TML) is a beta-adrenergic antagonist that blocks the aqueous humour secretion. However, simultaneous determination in one product brings about additional problems because both active pharmaceutical ingredients have to be quantified in one formulation. This means that both drug signals need to be usable under the same sample preparation and instrumental conditions [1–3].

Fixed-dose combination products create unique analytical challenges. Unlike liquid solutions for internal application, eye drops represent complex formulations that may require additional manipulation during dilution for measuring one compound while another remains stable in aqueous conditions. A suspension that contains BRZ should be carefully agitated and diluted before UV measurement. TML in contrast is routinely handled by aqueous response environment. Therefore, the

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same drug signals can display different analytical performance when present in one formulation. A method that can ensure linear response, quantification limits, consistent recoveries, and balanced formulation response is crucial for simultaneous quantification of BRZ and TML.

UV-visible spectrophotometry represents a widespread and relatively inexpensive instrument for pharmaceutical quality control. Other advantages of this platform include low operation costs, rapid response, small solvent amounts required for dilution, maintenance simplicity, and lack of chromatographic requirements. However, conventional absorption spectra analysis is prone to spectral interference and background shifts. The limitations of classical UV spectrophotometry can be addressed through the use of derivative UV procedures. The mathematical approach based on smoothing and differential operations proved to be valuable in spectroscopic signal analysis for decades [4–6].

Review of the literature suggests that a number of instrumental platforms can be employed for simultaneous quantification of CAR inhibitors and beta-blockers. Chromatography is a must-have procedure for impurity, degradation product, and instability study, as well as for separation of multi-component mixtures. Meanwhile, derivative UV spectrophotometry presents a useful option when precise and fast assay of a stable formulation is needed [7–9]. In particular, previous studies on the simultaneous determination of BRZ and TML confirm that spectrophotometric approaches can be valuable for the task, while the separative options are important for other purposes. This work explores whether numerical validation record for paired drug quantification by derivative UV is good enough for analytical control of fixed-dose products.

Modern quality assurance practice has shifted towards lifecycle thinking regarding analytical procedures. According to the latest guidance on method validation, a procedure should be fit-for-purpose, with validation criteria tailored for the intended use. Therefore, uncertainties, calibration, precision, and robustness must be assessed in the context of the actual decision that needs to be made [10–12]. This approach is particularly useful for validation of derivative UV procedures because high coefficient of determination does not guarantee suitability for routine assay. Lower working concentration has to be higher than the quantification limit, while recovery values have to be consistent throughout spiking levels. Besides, it is necessary to ensure that both drugs can be assayed with sufficient balance in one formulation. Thus, the connection of these values should be seen as the main quality assurance meaning.

Chemometrics provides various numerical approaches to transform and interpret analytical data depending on the actual problem to be solved. The current study does not require a complicated multivariate technique. Nevertheless, a chemometric approach to data interpretation involves reading calibration and validation values, recoveries, and formulation response in the context of one drug pair. A derivative UV procedure is characterized by two response channels, two limits of quantitation, two recovery curves, and the interdependence of both formulation results.

The concept of green analytical chemistry has transformed the evaluation process significantly. An analytical method is preferred only if it uses less solvent, produces less waste, requires less energy, and excludes toxic or hazardous substances. The twelve principles, eco-scale, analytical Green Index (AGREE), and greenness Assessment Procedure in ICH (GAPI) allow the environmental performance of a procedure to be discussed alongside separation capability or historical preferences [13–15]. In other words, claims about greenness are always supported with analytical validation.

The question that has been left open for BRZ and TML is whether ordinary numerical validation can be extended to paired formulation control. While the calibration and recovery of a derivative UV method may meet quality criteria, a routine laboratory will need to verify whether the two drug signals stay consistent in one product. Validation parameters like linearity, recovery, formulation assay percent, and greenness need to be discussed as a set of interconnected values.

The research question is formulated as follows: is it possible to perform a simultaneous and greener assay of BRZ and TML ophthalmic products based on derivative UV spectrophotometry with adequate calibration, recovery, balance, conformity, and greenness? The novelty lies in connecting the results for BRZ and TML into one analytical pair rather than keeping them as unrelated values. The numerical record is organized in accordance with five questions about binary calibration, recovery, formulation, conformity, and environmental impact.

2. Materials and methods

2.1. Analytical record and research design

Simultaneous determination of BRZ and TML in ophthalmic formulations has been performed through the use of first-order derivative UV spectrophotometry [16]. The analytical data included calibration ranges, derivative wavelengths, coefficients of determination, limits of detection and quantification, recovery values at 80, 100, and 120% spike concentrations, formulation assay percentages, and greenness values. The research design was based on the assumption that a proper analytical validation record can become relevant for paired binary product control.

Five main assay questions have been formulated to analyze the collected data. The first involved the discussion of calibration and sensitivity reserve. The second question related to recovery accuracy and repeatability at the mentioned spike levels. The third question concerned binary assay results in four commercial products, including three suspensions and one in situ gel. The fourth was concerned with product conformity based on the label claim deviation and formulation consistency. Finally, the fifth question was related to the environment-friendly performance of derivative UV spectrophotometry compared to one of the chromatographic platforms.

2.2. Instrumental basis

The first-order derivative UV was applied as an instrumental platform for simultaneous BRZ-TML measurement. BRZ was measured at 248.80 nm, while TML was measured at 297.60 nm. The two wavelengths served as derivative response points for both analytes. The linear ranges of calibration were set at 4–24 μg mL^{-1} for BRZ and 5–25 μg mL^{-1} for TML. The instrumental interpretation focused on linearity and lower sensitivity reserve compared to the working range of calibration.

2.3. Paired-assay suitability evaluation

The paired-assay suitability evaluation provided a way of interpreting validation values as binary drug pair values. Sensitivity reserve was evaluated as the relationship between the minimum calibration concentration and the corresponding LOQ value. The recovery values were discussed based on mean recovery percent, variation in replicates, and RSD%. The product binary formulation agreement was estimated based on the absolute difference between drug assay results. Deviation from the label claim was calculated in addition to support product conformity ranking.

The evaluation process outlined here is not an equivalent to the pharmacopeia or regulatory criteria of acceptability. However, it offers an opportunity to discuss the measurement values that are normally separated within different validation tables in a laboratory setting. The interpretation is consistent with the lifecycle approach to validation and measurement because the relationship between instrumental response, validation values, and intended use is highlighted [11, 12, 17].

2.4. Greenness assessment

Three greenness measures (AGREE value, analytical eco-scale score, and penalty points) were used for the comparison of derivative UV spectrophotometry with a chosen chromatographic technique. All three greenness values are based on different aspects of environmental and safety performance of the procedure. AGREE is based on the twelve principles of green analytical chemistry, eco-scale considers less desirable operational and chemical features, while penalty points estimate burden reduction [15, 18, 19]. A method was regarded as environmentally superior only in case of successful calibration and recovery performance and adequate formulation response.

3. Results and discussion

3.1. Calibration response and sensitivity reserve

The results of calibration are given in Table 1. The linear ranges of BRZ were 4–24 µg mL^{-1} with R^2=0.9998. As for TML, the linearity range was 5–25 µg mL^{-1} with R^2=0.9999. Limits of quantification were below the initial calibration standards for both compounds.
Calibration results draw a crucial distinction between the statistics on linearity and the actual assay margins for further operation. In other words, there are no statistically significant differences between the two correlation coefficients, yet the sensitivity reserves are considerably different because BRZ features a larger distance between the quantification limit and the lowest calibration point, while TML starts closer to its quantification limit.

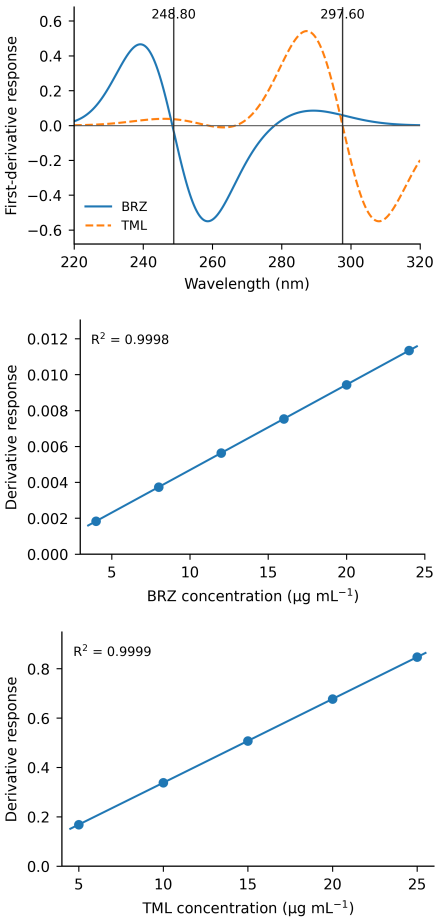

Figure 1: Derivative calibration.

The calibration diagrams reveal a clear relationship between the derivative measurement windows and concurrent quantification. The strong concentration-responsiveness ordering is observed for both BRZ and TML in their calibration plots, while the third panel provides the connection between the quantitative regression evidence and the decision made about the choice of UV measurements with derivatives.

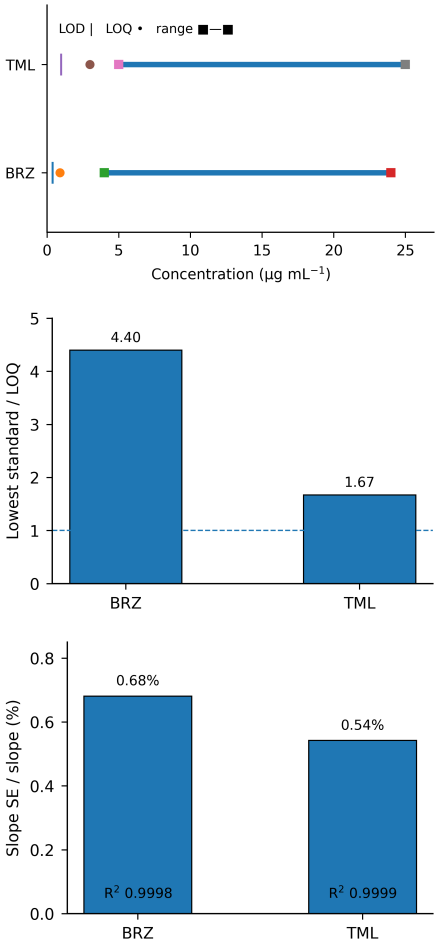

Figure 2: Sensitivity reserve.

These graphics are necessary to give additional meaning to the statistical validation evidence. The panel with limits and working ranges confirms that the two analytes have been analyzed within the ranges located above their LOQ values. Meanwhile, the panel illustrating the sensitivity reserve immediately reveals the difference in assay margins due to a narrower range for TML. Finally, the third panel shows that the difference in sensitivity reserve was not caused by any linearity issues since the correlations between concentrations and absorption were sufficiently strong in both cases. Therefore, the combination of these graphs will be very helpful in routine quality-control purposes as well as revealing potential risks related to an excessively ambitious dilution approach.

The calibration results allow for addressing the first half of the research question. Firstly, the analysis of data proves that first-order derivative UV spectrophotometry generates a sufficient linearity of response in order to perform simultaneous assay of BRZ and TML. Secondly, the paired-assay calibration allows us to conclude that the two drugs exhibit significantly different analytical sensitivity reserves despite being measured in equal conditions. It is important because decisions on fixed-dose assay depend on the ability of both components to provide sufficient reliability.

Table 1: Calibration evidence.

Analyte	Derivative wavelength	Linear range	R^2	Limit of detection	Limit of quantification	Sensitivity reserve
Brinzolamide	248.80 nm	4–24 µg mL^{-1}	0.9998	0.30 µg mL^{-1}	0.91 µg mL^{-1}	4.40
Timolol maleate	297.60 nm	5–25 µg mL^{-1}	0.9999	0.98 µg mL^{-1}	2.99 µg mL^{-1}	1.67

3.2. Recovery accuracy and repeatability

The recovery evidence is provided in Table 2. The BRZ mean recovery value is equal to 97.43% at the 80% spike level, 99.95% at the 100% spike level, and 103.22% at the 120% spike level. TML mean recovery is 101.73%, 103.34%, and 102.88% in the same cases. Relative standard deviation did not exceed 2%.

As it could be seen from the recovery table, the proposed derivative UV approach can be used for calibration in a biological matrix, and both drugs demonstrated reliable recoveries. Controlled repeatability was achieved for all spikes in the present study, while an insignificant growth of the BRZ recovery during the transition from low to high spiking indicates an acceptable concentration-dependent change in recovery. All three spikes of TML have the highest repeatability at the 120% spike level.

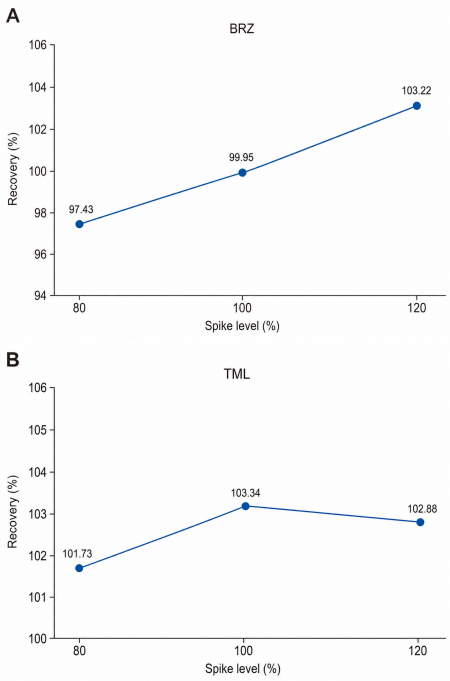

Figure 3: Recovery means.

It should be noted that there is no severe spike-level recovery problem concerning either analyte. Recovery of BRZ increases from the slightly low level at the 80% spike to the slightly higher level at the 120% spike, whereas TML consistently maintains the level above 100% within a fairly narrow range of mean values. Thus, this graphical visualization allows concluding that the presented approach provided the adequate accuracy of both components.

The replicates-recovery graphics represent a more strict view, compared with the mean-recovery graphs. Individual recovery points are still well-clustered for all spike levels, and the RSD profile proves controlled repeatability up to the 2% limit in the entire experiment. Importantly, a detailed assessment of the range panel allows proving that the recovery data were accurate, since the result is not affected by the presence of a favorable mean value.

Thus, the accuracy and repeatability results in relation to the recoveries answer the second part of the research question. The proposed

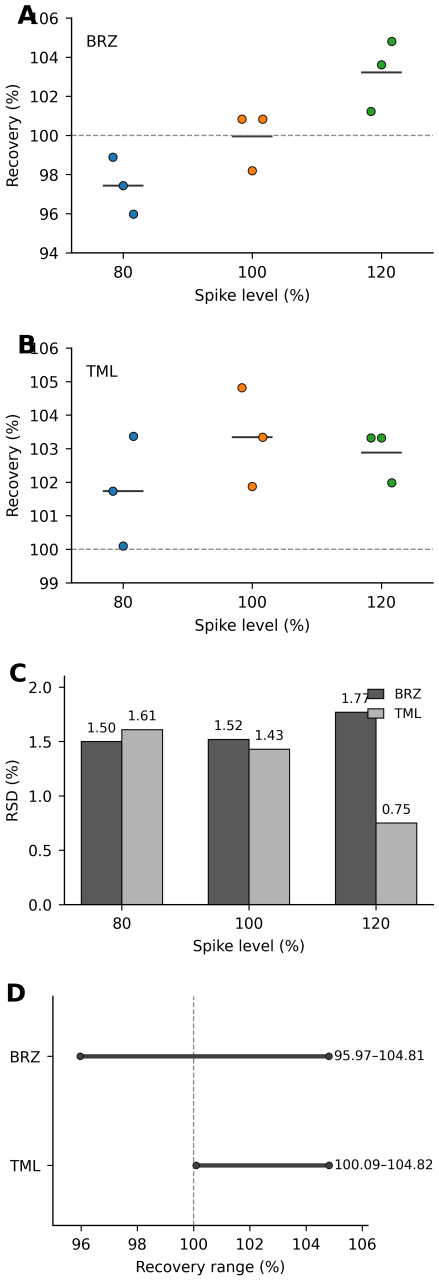

Figure 4: Recovery repeatability.

approach demonstrates acceptable recovery parameters for both active substances. It should be noted that this aspect is crucial when developing a fixed-dose assay since otherwise it will not be appropriate for a simultaneous quality control. Therefore, recovery consistency supports the applicability of the approach for paired assay purposes.

3.3. Formulation assay and binary agreement

Results of the product assays are represented in Table 3 with respect to three commercially available suspensions and one in situ gel. Assay

Table 2: Recovery evidence.

Analyte	Spiking level	Recovery entries	Mean recovery	Relative standard deviation
Brinzolamide	80%	98.89, 97.43, 95.97%	97.43%	1.50%
Brinzolamide	100%	100.83, 98.20, 100.83%	99.95%	1.52%
Brinzolamide	120%	101.23, 103.62, 104.81%	103.22%	1.77%
Timolol maleate	80%	101.731, 100.094, 103.367%	101.73%	1.61%
Timolol maleate	100%	104.816, 101.870, 103.343%	103.34%	1.43%
Timolol maleate	120%	103.323, 103.323, 101.984%	102.88%	0.75%

of BRZ varied from 102.63 to 105.39%, whereas TML assay values ranged from 102.52 to 105.68%.

The formulation table provides product-specific interpretation of the validation results. Values of S1, S2, S3, and ISG are relatively similar to the claimed content, but their binary agreement is different. ISG exhibits the tightest paired agreement with just 0.03 percentage-point separation of BRZ and TML, and S2 also demonstrates a strong pair of assay results. In contrast, S3 exhibits the largest separation of analytes, as BRZ content exceeds its counterpart's TML. The distinction is important since the fixed-dose quality control implies the correlation between these two assay results.

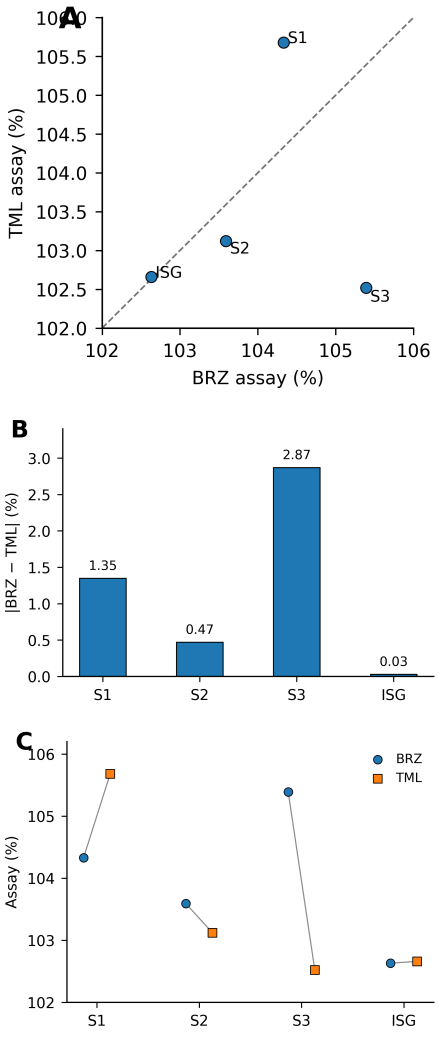

Figure 5: Formulation balance.

The formulated-balance charts illustrate the principle of paired assay. As follows, the paired chart illustrates the proximity of each formulation to the equal response for BRZ and TML, the difference chart highlights the largest separation of these analytes, and the assay values of all four formulations stay within the acceptable range. Therefore, the visual analysis helps to differentiate between individual assaying acceptability and paired formulation balance.

The results provide an answer to the third part of the research question. Firstly, the derivative UV spectrophotometry technique can be used both for the commercial suspensions and the in situ gel. Secondly, the paired assay allows identifying which products demonstrate the best binary agreement. Such approach is valuable for product differentiation even if two products are equally close to the claimed content.

3.4. Conformity ranking for routine decisions

The conformity ranking provided in Table 4 translates formulation analysis into a quality control routine. The ranking considers both deviation of individual products from the claim and paired assay separation as analytical criteria. The tool is not regulatory standardization and cannot be viewed as one, and its purpose is to assess the relative analytical balance of formulations being tested.

This ranking system summarizes the assay-product values in a useful laboratory sequence. ISG ranks first since it has both the minimum pair difference and minimum total deviation load. S2 ranks second since it has high binary conformity. S3 ranks third after S2 because of the strong separation of the two components, despite both having assay percent values near the claims. S1 ranks fourth due to its high total deviation load compared to other products.

Conformity map confirms that there is a valid reason for such ranking. While rank panel shows ordering of the products, the combined deviation and paired difference panels show the rationale behind such ordering in terms of analytics. Such representation is convenient in quality control report since it avoids having a single assay percentage being dominant while instead focusing on the fixed-dose nature of the pair.

Conformity assessment allows expanding this methodology beyond regular validation reporting. Calibration and recovery data prove that the measurement is done properly, while conformity ranking reveals the use of the assay to support comparison of products. This is significant in case of routine measurements since an analyst may be required to compare different formulations or products while still satisfying general assay requirements.

3.5. Greenness profile and environmental preference

Results of greenness comparison are shown in Table 5. AGREE value was increased from 0.62 to 0.77 and analytical eco-scale score was raised from 77 to 88 by switching to the derivative UV-based system. Total number of penalty points was also reduced from 23 to 12, resulting in 47.83% decrease.

It can be seen from the greenness table that the environmental preference cannot be reduced to just one number. The AGREE scale, the eco-scale score, and the penalty points indicate a preference for the derivative UV method in each individual case. The convergence of the preferences is important because each tool reflects the different aspects of environmentally preferable analysis, such as the use of solvents, haz-

Table 3: Formulation evidence.

Formulation	Brinzolamide assay	Timolol maleate assay	Brinzolamide deviation	Timolol deviation	BRZ–TML difference
S1	104.33%	105.68%	4.33 percentage points	5.68 percentage points	1.35 percentage points
S2	103.59%	103.12%	3.59 percentage points	3.12 percentage points	0.47 percentage points
S3	105.39%	102.52%	5.39 percentage points	2.52 percentage points	2.87 percentage points
ISG	102.63%	102.66%	2.63 percentage points	2.66 percentage points	0.03 percentage points

Table 4: Conformity ranking.

Rank	Formulation	Interpretation
1	ISG	Closest paired agreement and lowest combined deviation from label claim
2	S2	Strong binary agreement with modest individual deviations
3	S3	Acceptable individual assay values but the largest BRZ–TML separation
4	S1	Acceptable individual assay values with the highest combined deviation

Table 5: Greenness evidence.

Method class	AGREE value	Eco-scale score	Penalty points	Interpretation
Derivative UV spectrophotometric platform	0.77	88	12	Lower-burden routine assay option
Chromatographic platform	0.62	77	23	Higher solvent and operational burden

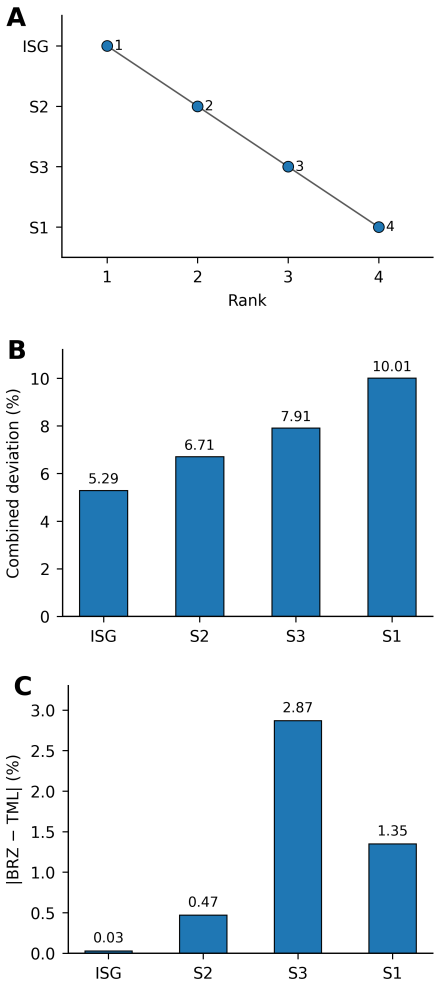

Figure 6: Conformity map.

ards, waste generation, operation complexity, and adherence to green analytical chemistry principles. Therefore, the greenness conclusion is supported by the convergence of opinions about the environmental advantage.

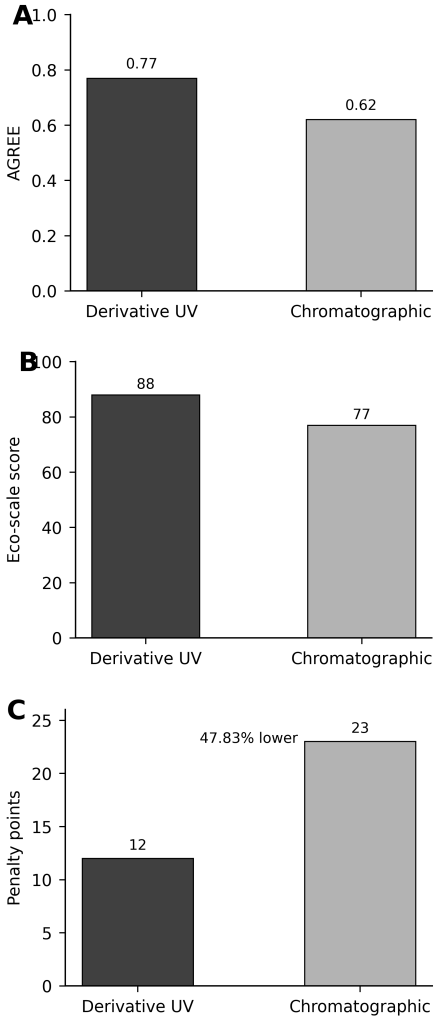

Figure 7: Greenness comparison.

Thus, the visual representation of greenness shows a clear trend towards a favourable environmental profile. It is especially important because environmentally preferable analysis should be proved by converging evidence. The green conclusion receives additional weight because in this case, the same favourable conclusion has been reached in all three greenness assessment methods.

It should also be noted that the conclusions obtained on the environmental profile should be considered along with the other pieces of evidence. If the analytical method was environmentally advantageous but compromised one of the main criteria of the assay task, it could not be considered suitable. The current evidence does not have such drawbacks since the derivative UV platform shows favourable results in terms of linearity, quantifiable working ranges, recovery, and assay product performance.

3.6. Integrated assay assessment

The results of the main validation and the environmental impact assessment are summarized in Table 6. Although the decision score of 0.750 is regarded as the internal assay suitability index, its primary importance is in demonstrating that different evidence domains support the same conclusion.

Table 6: Assay evidence.

Assessment domain	Main finding	Analytical meaning
Calibration response	R^2=0.9998 for BRZ and 0.9999 for TML	Derivative response remained strongly linear in both working ranges
Sensitivity reserve	LOQs were below the lowest calibration standards	Both assay channels began above quantifiable concentration limits
Recovery coherence	Mean recovery stayed between 97.43 and 103.34%	Accuracy and repeatability were suitable for simultaneous assay
Product applicability	Assay values ranged from 102.52 to 105.68%	The procedure worked across suspensions and the in situ gel
Binary agreement	ISG showed a 0.03 percentage-point BRZ–TML difference	The best tested product profile had nearly identical paired assay response
Greenness	AGREE 0.77, eco-scale 88, and 12 penalty points	The method had a lower environmental burden than the chromatographic comparison
Overall assay suitability	Integrated score of 0.750	The method is suitable for routine paired BRZ–TML assay decisions

Integration of results in the form of assay table answers the research question about the feasibility of the proposed derivative UV platform. Calibration provides response proportionality, sensitivity reserve preserves the lower part of the working range, recovery demonstrates matrix-appropriate accuracy, product assay shows applicability of the method to the formulation type, binary agreement shows fixed-dose balance, and greenness confirms preference of the environment-friendly option. This synthesis allows concluding about the superiority of the combination over a single parameter due to the connection of analytics and the decision-making.

The graphical summary of the integrated assay profile helps to understand how the numerical validation data supports the final decision. The score panel shows overall method appropriateness, linearity panel highlights strong regressions, validation interval panel demonstrates recovery and product assay values with respect to the 100% reference line, and greenness change panel reflects the improvement of the environmental friendliness. Based on these considerations, it is possible to state that the derivative UV technique is not just an inexpensive alternative to chromatographic analysis but an analytically interpretable and environmentally preferable assay technique for a fixed-dose product class.

At the same time, these integrated conclusions define the boundaries of this study. This investigation confirms the suitability for routine assay and comparative formulation interpretation; however, it cannot substitute impurity profiling, forced degradation studies, and full stability-indicating chromatography when necessary. Additional studies of placebo interference, intra-analyst precision, inter-instrument precision, robustness, and batch independence could help improve method transferability. Consistent with a fit-for-purpose approach to method development, the technique is adequately developed for routine

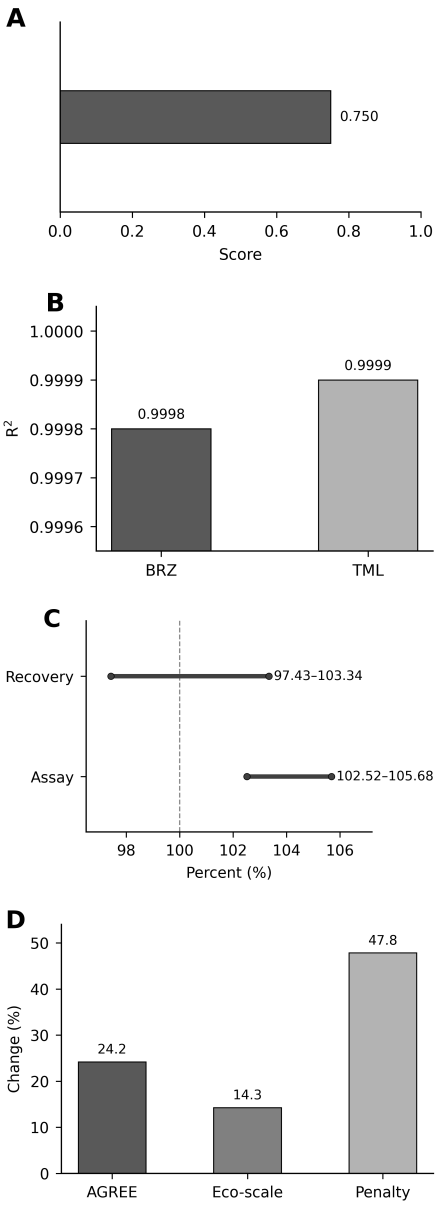

Figure 8: Integrated assay profile.

BRZ-TML assay and further applications may need chromatographic support.

4. Conclusion

This work has considered the possibility of developing a reliable and environmentally friendly method of paired assay for BRZ–TML ophthalmic products when taking into account calibration reserve, recovery behaviour, formulation agreement, and solvent consumption. The answer is positive. First-order derivative UV analysis demonstrated strong linearity of both analytes with R^2=0.9998 for BRZ and R^2=0.9999 for TML. Both working ranges started higher than the limits of quantification, providing wider sensitivity reserve for BRZ and narrower but satisfactory one for TML. Therefore, it was established that this analytical procedure provides sufficient instrumental base for simultaneous estimation, identifying the TML channel as the most sensitive response.

Moreover, recovery and product assay results confirmed applicability of the method beyond calibration standards. For six groups of recovery, average recovery was within 97.43–103.34%; all relative standard

deviations were lower than 2%. Assay of three commercial suspensions and one in situ gel demonstrated satisfactory agreement with label claims for both drugs; the highest binary agreement was observed for the latter, being 0.03 percentage point; maximum paired separation for S3 was 2.87 percentage point. These results indicate applicability of the derivative UV procedure to assay and paired fixed-dose estimation. Finally, the presented evidence has positively answered the question on environmental performance of the method. The derivative UV method achieved better greenness scores than HPLC in AGREE value (0.77), eco-scale score (88), and number of penalty points (12). Thus, the conclusion is specific to the tested BRZ-TML assay method: first-order derivative UV spectrophotometry can be used as a solvent-saving paired quality control method when calibration reserve, recovery behaviour, formulation agreement, and greenness are evaluated together.

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