

Beyond Conventional RP-HPLC: Quality-by-Design, Green Analytical Chemistry, and Regulatory Perspectives for Multicomponent Analysis of Antihypertensive Drug Combinations.

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Abstract

Multicomponent analysis of antihypertensive drugs sits at the intersection of pharmaceutical quality control, bioanalysis, pharmacokinetics, and clinical therapeutic drug monitoring (TDM). Conventional reversed-phase high-performance liquid chromatography (RP-HPLC) remains useful because of its broad availability and flexible detection options; however, a method optimized for a synthetic mixture or tablet extract cannot automatically be transferred to plasma or serum, where endogenous components, concomitant medicines, protein binding, and low analyte concentrations can compromise selectivity and quantitative reliability. At the same time, method development is increasingly expected to demonstrate scientific understanding, robustness, lifecycle control, and reduced environmental burden. This review critically integrates analytical quality by design (AQbD), green analytical chemistry (GAC), chromatographic selectivity, sample preparation, detection, and regulatory expectations into a purpose-led framework for multicomponent antihypertensive analysis. Published bioanalytical examples demonstrate that LC-MS/MS can support targeted measurement of nebivolol in human plasma and simultaneous quantification of a broad panel of antihypertensive drugs in serum. These studies establish feasibility for their stated purposes, but they do not establish that one universal method is suitable for every drug, matrix, concentration range, or clinical question. ICH Q14 and Q2(R2) provide a science- and risk-based framework for analytical procedure development and validation, whereas ICH M10 specifically addresses bioanalytical methods and study-sample analysis. GAC metrics such as AGREE, GAPI, and Analytical Eco-Scale can make environmental trade-offs more transparent, although their outputs are not interchangeable and do not replace assessment of analytical fitness. The review proposes an integrated decision pathway linking intended use, analytical target profile, risk-based experimentation, matrix-appropriate validation, sustainability assessment, and lifecycle monitoring. The principal research need is transparent comparative evidence that evaluates analytical performance and environmental burden together without sacrificing the reliability of pharmacokinetic or TDM conclusions.

Keywords: Antihypertensive Drugs; Bioanalytical Chromatography; Analytical Quality By Design; Green Analytical Chemistry; Method Validation.

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1. Introduction

Antihypertensive therapy commonly involves agents from several pharmacological classes, including β -adrenoceptor blockers, calcium-channel blockers, angiotensin-converting-enzyme inhibitors, angiotensin II receptor blockers, and diuretics. A patient may receive more than one agent, and drug-development studies may require measurement of parent compounds, active metabolites, or multiple prescribed medicines in a single biological sample. Reliable analytical measurement therefore supports several distinct activities: pharmacokinetic (PK) studies, bioavailability and bioequivalence studies, drug-drug interaction investigations, therapeutic drug monitoring (TDM), adherence assessment, and research into unexplained treatment failure. These activities share analytical technologies but do not share identical analytical requirements. The concentration range needed for a PK

profile may differ from that needed for an adherence screen; a panel intended to detect exposure to several medicines is not necessarily optimized to quantify each compound with the precision needed for a regulatory PK endpoint [1].

RP-HPLC with ultraviolet (UV) or diode-array detection is an established approach for compounds with sufficient absorbance and when the sample matrix and concentration range permit adequate selectivity and sensitivity. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is particularly useful for low-level measurements in complex matrices and for panels containing compounds that are difficult to distinguish by UV detection alone. For example, Gundersen and colleagues developed a UHPLC-MS/MS method for quantification of 21 antihypertensive drugs in serum for routine TDM [1]. Nandania and colleagues reported an LC-MS/MS method for nebivolol in human

plasma with a calibration range of 50-5000 pg/mL and a reported sensitivity of 30 pg/mL [2]. The two examples are valuable but answer different analytical questions: one addresses a broad serum panel, whereas the other targets a single analyte for quantitative bioanalysis. They should not be ranked as if their analyte scope, calibration range, or intended use were equivalent.

The analytical challenge becomes more complex when several chemically dissimilar antihypertensive agents are measured together. Differences in ionization, polarity, protein binding, extraction behaviour, chromatographic retention, and effective circulating concentrations can create conflicting method requirements. A common sample-preparation procedure may recover some analytes efficiently while losing others; a single chromatographic condition may retain one compound adequately but cause early elution or excessive retention of another. In LC-MS/MS, co-eluting endogenous components can suppress or enhance ionization, and transitions can be affected by isobaric compounds, in-source fragmentation, carryover, or cross-talk. These issues mean that a method with acceptable calibration and precision in neat standards may still be unsuitable for clinical samples unless matrix-specific performance has been demonstrated [4,5].

A second challenge is the changing expectation for analytical method development. Analytical quality by design (AQbD) encourages method developers to define intended performance in advance, assess risk, use structured experimentation where justified, and establish a control strategy. ICH Q14 describes science- and risk-based approaches to analytical procedure development, while ICH Q2(R2) describes principles for validating analytical procedures used in pharmaceutical quality contexts [3,4]. For bioanalytical methods supporting regulated nonclinical or clinical studies, ICH M10 provides more directly relevant expectations for validation and study-sample analysis [5]. These guidelines are complementary rather than interchangeable: choosing the correct framework depends on whether the method is used for release/stability testing, exploratory bioanalysis, TDM, or regulated PK studies.

A third challenge is sustainability. Analytical workflows consume solvents, sample-preparation reagents, extraction cartridges, vials, filters, electricity, and instrument time, and they generate chemical and plastic waste. Green analytical chemistry (GAC) encourages prevention or reduction of such burdens while maintaining fitness for purpose. Tools including the National Environmental Methods Index (NEMI), Analytical Eco-Scale, Green Analytical Procedure Index (GAPI), and Analytical Greenness Calculator (AGREE) evaluate different aspects of analytical greenness [6-9]. Because their scoring principles and system boundaries differ, a score from one metric should not be directly compared with a score from another. Moreover, a greener

method is not a useful substitute if it fails to quantify the analyte reliably at the required concentration.

The central proposition of this review is that multicomponent antihypertensive analysis should be designed as a linked decision system rather than as a chromatographic optimization exercise. The sequence begins with intended use and an analytical target profile, proceeds through risk assessment and method development, establishes performance in the relevant matrix, evaluates environmental and operational burdens, and continues through lifecycle monitoring. The review focuses on RP-HPLC and related liquid-chromatographic platforms for multicomponent analysis, with bioanalytical examples used to define the boundary between formulation analysis and biological measurement. Routine assay of synthetic mixtures or finished tablets is not treated as equivalent to bioanalysis. The contribution is a critical synthesis of the connections among AQbD, GAC, validation, and use-case suitability, with emphasis on the limits of transferring methods between matrices and purposes.

2. Scope, Evidence Approach, and Analytical Context

2.1 Scope and evidence boundaries

This review is a critical narrative synthesis rather than a systematic review or meta-analysis. The evidence base emphasizes authoritative regulatory documents, methodological papers on analytical development and greenness assessment, and representative primary studies relevant to antihypertensive bioanalysis. Regulatory claims are grounded in the relevant ICH or agency guidance, and quantitative details are included only when reported by the cited source. Because studies vary in analyte panel, biological matrix, instrument configuration, sample preparation, and intended use, numerical results are not pooled and are not interpreted as a head-to-head comparison. The available examples are used to illustrate design principles, not to claim comprehensive coverage of every published antihypertensive assay [6].

A publication-ready systematic review would require a reproducible database search, explicit inclusion/exclusion criteria, duplicate screening, and a structured extraction sheet covering analytes, matrix, sample preparation, detector, calibration range, LLOQ, accuracy, precision, matrix effects, recovery, stability, run time, solvent consumption, and validation framework. The present manuscript does not claim that such a complete systematic search has been performed. Instead, it identifies an integrated conceptual framework and specifies what future comparative studies should report. This distinction matters because a narrative review should not imply that the absence of a paper from the selected examples proves that no such paper exists [7].

2.2 Distinguishing formulation analysis from bioanalysis

A synthetic mixture or dosage-form extract is generally more controlled than plasma or serum. In a synthetic

mixture, the analyst may know the composition and can often use a solvent that dissolves all target analytes. In a tablet extract, excipients may interfere, but the matrix is usually not the complex endogenous mixture encountered in blood-derived samples. Plasma and serum introduce endogenous proteins, phospholipids, salts, metabolites, and potential co-medications. Consequently, validation parameters and experimental designs must reflect the intended matrix. Demonstrating simultaneous assay of nebivolol hydrochloride, telmisartan, and cilnidipine in a synthetic mixture may establish formulation-method performance, but it does not establish suitability for TDM or PK analysis without separate matrix-specific development and validation [8, 9].

The distinction is especially important for the selected journal. *Journal of Chromatography B* emphasizes separation science relevant to biological and biomedical research, including chromatography, sample preparation, detection, mass spectrometry, and data handling [10]. A review focused only on routine quality-control assay of a three-drug synthetic mixture may therefore have a weaker fit than a review that critically addresses biological matrices, analytical selectivity, and the consequences of method performance for biomedical measurements. The current framework retains multicomponent antihypertensive analysis as the core topic but makes the intended-use and matrix distinction explicit throughout.

3. Analytical target profile and technology selection

3.1 Define the intended decision before choosing the instrument

The analytical target profile (ATP) should describe the measurement objective in terms that can be tested: analyte(s), matrix, reportable concentration range, required selectivity, acceptable accuracy and precision, sample throughput, and the decision supported by the result. For a regulated PK study, the ATP must support reliable concentration-time data across the relevant sampling window, including concentrations near the lower end of the profile. For TDM, the ATP should reflect the expected concentration range and the clinical context in which the result is interpreted. For adherence assessment, the goal may be detection of recent exposure to multiple medicines, but detection alone does not prove

Table 1. Intended use should determine matrix, analytical technology, validation emphasis.

Intend ed use	Typical matrix	Primary analytica l need	Impor tant risks	Evidenc e expected
Finishe d-product assay	Tablet/capsule extract	Assay, specificit y, range, stability-indicating capability where relevant	Excipi ent interference, degrad ation produc ts,	Procedur e validatio n suited to product quality

that the prescribed dose was taken at the correct time or that exposure was therapeutically adequate [11].

The ATP should also identify constraints such as sample volume, available instrumentation, analysis turnaround, number of analytes, stability requirements, and the likely presence of metabolites or co-medications. Without these constraints, optimization can be misdirected. For example, maximizing the number of compounds in a panel may create unnecessary compromises in sensitivity or selectivity if only a small subset is needed for the clinical question. Conversely, running separate methods for each drug may increase sample use, solvent consumption, and turnaround time when a validated multi-analyte method could meet all required performance criteria.

3.2 Choosing UV, fluorescence, LC-MS/MS, or high-resolution MS

UV or diode-array detection remains attractive because the instrumentation is comparatively accessible and the data can be straightforward to interpret. Its suitability depends on analyte absorbance, matrix background, chromatographic resolution, and the concentration range. Fluorescence detection can improve sensitivity for naturally fluorescent compounds or derivatives, but derivatization adds reagents, time, and possible sources of variability. LC-MS/MS is often advantageous for biological matrices because mass transitions add selectivity and sensitivity; however, it requires careful assessment of ion suppression, source contamination, transition specificity, and instrument maintenance. High-resolution MS may support broad screening or retrospective analysis, but its use for quantitative purposes still requires appropriate calibration, selectivity, precision, and validation evidence.

Instrument choice should therefore be treated as a decision based on required performance, not a simple hierarchy in which a more sophisticated detector is always better. A robust UV method may be suitable for a formulation assay, while LC-MS/MS may be justified for plasma concentrations that are too low or too matrix-sensitive for UV detection. The same active ingredient may require different analytical procedures for release testing, bioequivalence, and TDM. This is not inconsistency; it reflects different analytical target profiles [12] (Table 1).

			extract ion bias	control [3,4]
PK / bioequivalence	Plasma or serum	Reliable quantification across concentration-time profile	Matrix effects , stability, carryover, LLOQ , dilutio	Bioanaly tical validation and study-sample controls [5]

			n integrity	
TDM	Serum/plasma	Fit-for-purpose concentration measurement for clinical interpretation	Co-medication, variable sampling time, matrix differences	Validated selectivity, calibration, precision and appropriate clinical interpretation [1,5]
Adherence	Serum/plasma; someti	Detect exposure to	Detect ion window	Validated panel performance

assessment	mes other matrices	selected prescribed agents/metabolites	w, metabolism, dose timing, panel coverage	nence; no overinterpretation of a single result [1]
Exploratory screening	Biological matrix	Broad coverage and analyte identification	False positives, isobars, retrospective identification limits	Fit-for-purpose confirmation and transparent identification confidence

4. A QbD-based development of multicomponent chromatographic methods

4.1 CQA and risk assessment

AQbD begins by translating the ATP into critical method attributes (CMAs). Depending on intended use, these may include selectivity, accuracy, precision, reportable range, lower limit of quantification (LLOQ), carryover, recovery, matrix effect, analyte stability, retention time, peak shape, resolution, run time, and robustness. A CMA is not simply a parameter that can be measured; it is a performance attribute whose failure would compromise the intended analytical decision. For example, an assay used to quantify low plasma concentrations may prioritize LLOQ and matrix effect, whereas a high-throughput panel may give additional weight to carryover, injection-to-injection reproducibility, and cycle time [13].

Risk assessment should identify method variables that may affect these attributes. Chromatographic variables include stationary-phase chemistry, column dimensions, mobile-phase pH, organic fraction, buffer concentration, gradient profile, flow rate, column temperature, and injection volume. Sample-preparation variables may include extraction pH, organic-to-aqueous ratio, mixing time, centrifugation, sorbent chemistry, wash composition, and elution volume. For LC-MS/MS, source temperature, gas settings, ionization mode, collision energy, dwell time, and transition selection may also be critical. Risk tools such as cause-and-effect diagrams or failure mode and effects analysis can prioritize experiments, but their scores are hypothesis-generating; they do not prove that a factor is critical until evidence supports the relationship [14].

4.2 Design of experiments and interaction effects

One-factor-at-a-time optimization can be useful for early scouting, but it is inefficient for evaluating interactions among parameters. In multicomponent analysis, interactions are plausible: pH may affect analyte ionization and retention; organic fraction may affect both

elution and electrospray response; extraction pH may influence recovery of basic and acidic drugs in different directions; and gradient slope may interact with flow rate and column temperature. Factorial designs can screen many variables with fewer experiments than exhaustive testing, while response-surface designs can characterize the most influential factors and identify operating regions that meet predefined criteria [15].

The statistical model should be assessed for residual structure, lack of fit, predictive error, and practical interpretability, not only for a high coefficient of determination. A high R^2 can occur even when a model predicts poorly near the edges of the design space. Confirmation experiments should be performed at the selected operating condition and, when relevant, at boundary conditions. The response variables should represent method performance directly—such as resolution, matrix effect, recovery, or precision—rather than convenience metrics alone. A desirability function can assist multi-response optimization, but the selected solution must still satisfy each mandatory performance criterion [16].

4.3 Method operable design region and control strategy

ICH Q14 describes science- and risk-based development, including use of multivariate experiments and establishment of parameter ranges or a method operable design region (MODR), where appropriate [17]. A MODR represents a region in which method performance has been demonstrated to remain acceptable, rather than a single software-generated optimum. Establishing a MODR requires a sufficiently informative design, defined acceptance criteria, model evaluation, and confirmation experiments. It may be unnecessary for a simple low-risk procedure; the approach should be proportionate to method complexity and risk.

The control strategy should specify routine set-points, permitted ranges, system-suitability criteria, calibration checks, column-equilibration requirements, reagent and

mobile-phase preparation, and actions for out-of-control results. A method can be statistically optimized yet operationally fragile if it depends on a narrow pH window, a specific column lot, or tightly controlled room conditions. Robustness studies should therefore reflect realistic variations in laboratory practice and instrument performance. Method transfer and lifecycle monitoring should be planned during development rather than treated as an afterthought.

5. Sample preparation: analytical recovery and environmental burden

5.1 Protein precipitation, liquid-liquid extraction and solid-phase extraction

Sample preparation can determine both analytical performance and environmental burden. Protein precipitation is simple, rapid, and compatible with high throughput, but may leave phospholipids and other matrix constituents that contribute to ion suppression or contaminate the analytical system. Liquid-liquid extraction can improve cleanup and selectivity, yet commonly involves organic solvents, phase separation, evaporation, and reconstitution. Solid-phase extraction can provide selective cleanup and concentration, but requires sorbent cartridges or plates, conditioning, washing, elution, and additional consumables. No extraction technique is inherently best for all antihypertensive drugs because the compounds differ in physicochemical properties and the intended panel may include drugs with contrasting extraction behavior [18]. Miniaturized extraction, dispersive sorbent approaches, microextraction, and reduced-volume formats may lower solvent consumption and sample requirements. Their practical value depends on extraction reproducibility, sorbent availability, throughput, operator handling, and whether the technique can be transferred between laboratories. A microextraction procedure that uses very little solvent but produces variable recovery may require repeat analysis and thereby increase total waste. Sustainability must therefore be assessed at the workflow level, including repeat runs, quality-control failures, and consumables, rather than only at the nominal solvent volume of a successful injection [19].

5.2 Recovery, matrix effects and internal standards

Recovery and matrix effect are distinct. Recovery describes the fraction of analyte extracted from the sample; matrix effect describes the influence of co-extracted components on measurement response, particularly ionization in LC-MS/MS. Quantitative methods do not always require complete recovery, but recovery should be sufficiently consistent and the overall method should meet accuracy and precision requirements. A high recovery value does not demonstrate the absence of ion suppression, and a low but consistent recovery may still be compatible with reliable quantification when calibration and internal-standard correction are appropriate [20].

Matrix-effect evaluation should consider multiple independent sources of matrix and should reflect the

population and sample types likely to be encountered. Haemolysis, lipemia, co-administered medicines, and endogenous variability may be relevant depending on the intended use. Stable-isotope-labelled internal standards often provide strong compensation for extraction and ionization variability when they co-elute and behave similarly to the analyte. However, they cannot automatically correct interference from a compound that shares a transition or a chromatographic retention window, nor can they rescue poor sample handling or inadequate selectivity. These factors must be tested, not assumed [21].

5.3 Sample volume, storage and pre-analytical variation

Sample volume can be a practical constraint in paediatric studies, small-volume clinical sampling, or studies with repeated time points. Lowering sample volume may reduce the burden on participants and reduce reagent consumption, but it can also challenge sensitivity and precision. Storage temperature, duration, freeze-thaw cycles, bench-top exposure, processed-sample stability, and autosampler residence should be matched to the actual study workflow. A method validated only under ideal storage conditions may not be reliable when samples are transported between sites or analysed in batches.

Pre-analytical metadata should be retained where it can influence interpretation: collection time, time since dose, sample matrix, storage history, and relevant handling deviations. For PK or TDM applications, a precise chromatographic result cannot compensate for an incorrectly timed or mishandled specimen. The analytical workflow should therefore be considered from collection through reporting, not merely from injection onward [22].

6. Separation science and detection for multicomponent antihypertensive analysis

6.1 Stationary phase, mobile phase and retention control

C18 reversed-phase columns are a common starting point, but stationary-phase selection should be driven by the actual selectivity problem. Phenyl, polar-embedded, cyano, and mixed-mode phases may provide alternative selectivity when compounds co-elute or exhibit unsuitable retention. Particle size and column dimensions affect efficiency, pressure, and run time. UHPLC can shorten analysis and improve efficiency, but the higher pressure requirements, instrument configuration, and column lifetime should be included in practical and sustainability comparisons. A shorter chromatographic run is beneficial only when resolution, sensitivity, carryover, and reproducibility remain acceptable [23].

Mobile-phase pH and buffer composition affect analyte ionization, stationary-phase interactions, peak shape, and detector compatibility. Nonvolatile buffers that are acceptable for UV detection may be unsuitable for direct MS coupling because of source contamination or ionization effects. Conversely, volatile additives

compatible with MS may not provide the same selectivity as a buffer system developed for UV detection. A detector change therefore usually requires method redevelopment, not merely replacing the detector while leaving all other conditions unchanged [24].

6.2 Gradient and isocratic elution

Isocratic elution can be robust and easy to transfer when analytes have a relatively narrow retention range. Gradient elution can accommodate chemically diverse panels and can assist in removing retained matrix components. Its disadvantages include equilibration time, dwell-volume sensitivity, and possible differences between instruments. A gradient method's total cycle time should include the analytical gradient, wash, re-equilibration, and any blank or carryover-control injections. Reporting only the nominal gradient duration understates the resource use and may lead to misleading throughput comparisons.

6.3 LC-MS/MS selectivity and panel design

LC-MS/MS adds mass-selective detection but is not immune to interference. Potential problems include isobaric compounds, metabolites that share transitions, in-source fragmentation, cross-talk between transitions,

Table 2. Technology choices should be judged against the analytical target profile

Method feature	Potential advantage	Main limitation / risk	What should be reported
RP-HPLC-UV/DA D	Accessible instrumentation; useful for compounds with adequate UV response	Matrix background and limited sensitivity/selectivity for low-level bioanalysis	Matrix, wavelength, specificity evidence, range, extraction, chromatographic resolution
LC-MS/MS	High sensitivity and selectivity; supports multi-analyte quantification	Ion suppression, transition interference, carryover, instrument complexity	Transitions, internal standards, matrix-effect assessment, carryover, calibration and validation

7. Validation and regulatory interpretation

7.1 ICH Q14 and Q2(R2): development and validation of analytical procedures

ICH Q14 and ICH Q2(R2) were adopted as harmonized guidelines in 2023 and became effective in the European Union on 14 June 2024 [3,4]. Q14 describes science- and risk-based development of analytical procedures, including prior knowledge, risk assessment, multivariate

ion suppression/enhancement, and carryover after highly concentrated samples. Qualifier transitions, ion ratios, retention-time windows, blank matrix testing, and system suitability should be applied where appropriate to the method and instrument. Dwell times and scheduled acquisition windows must provide enough data points across chromatographic peaks without sacrificing sensitivity for less responsive analytes [25].

Multi-analyte panels should be designed around a clinically or scientifically justified analyte list. Adding an analyte may require changes to chromatography, extraction, calibration, internal-standard strategy, and validation. A panel that contains many drugs but performs poorly for a subset may be less useful than two smaller methods with clearly defined performance. The published serum assay for 21 antihypertensive drugs demonstrates that broad TDM panels are feasible [26], while the targeted nebivolol method demonstrates a separate route to sensitive single-analyte quantification [27]. Neither study establishes that a single universal chromatographic method can quantify all antihypertensive agents across all biological matrices.

UHPLC	High efficiency and potentially shorter runs	Higher pressure; system and column compatibility; transfer differences	Total cycle time, pressure, column specification, equilibration, robustness
Miniaturized sample preparation	Potentially lower sample and solvent use	May increase variability or require specialized consumables	Recovery, precision, matrix effects, solvent and consumable inventory
High-resolution MS	Broad screening and potential retrospective analysis	Data complexity; quantitative performance is application-specific	Mass accuracy, identification criteria, calibration, validation and confirmation strategy

experiments where justified, robustness, parameter ranges, and lifecycle management. Q2(R2) describes principles for validation and evaluation of performance characteristics to demonstrate that an analytical procedure is fit for its intended purpose. Together they support a lifecycle view in which development knowledge can inform validation and subsequent change management [28].

The scope of Q2(R2) is primarily analytical procedures used for release and stability testing of drug substances and products, although its scientific principles may be applied in a phase-appropriate way to some procedures used during clinical development [29]. This scope matters for the present review: it would be inaccurate to treat Q2(R2) as the sole or universal validation guideline for measuring drug concentrations in plasma or serum. Pharmaceutical product assay, impurity testing, and bioanalytical concentration measurement overlap in basic analytical concepts but differ in matrix-related risks and study-sample requirements.

7.2 ICH M10 for bioanalytical methods

ICH M10 addresses validation of bioanalytical methods and analysis of study samples for chemical and biological drugs and their metabolites in biological matrices, including nonclinical and clinical studies supporting regulatory decisions [30]. It emphasizes that a method must be adequately characterized, validated, and documented for its intended purpose. For

7.3 Method transfer, changes and lifecycle monitoring

Validation is a point-in-time demonstration of performance under defined conditions; it does not eliminate future risk. Columns, instruments, software, reagent suppliers, internal-standard lots, extraction consumables, and laboratory practices can change. A lifecycle approach records the method's knowledge base, critical parameters, operating ranges, system-suitability criteria, change history, and response to performance drift. Method transfer should examine equipment differences, matrix sources, analyst technique, calibration preparation, and local environmental conditions. For multi-site studies, transferability is not proven solely by obtaining similar retention times; the **Table 3. Bioanalytical validation - biological matrix, intended decision, and governing guidance [5].**

Attribute	Why it matters in bioanalysis	Example of evidence / control
Selectivity	Endogenous components and co-medications can mimic or obscure analyte response	Blank matrices, relevant interferences, retention/transition criteria
Calibration and range	Concentration estimates must be reliable over the intended study range	Matrix-matched calibration, justified model/weighting, QC samples
Accuracy and precision	Bias and variability affect PK parameters and clinical interpretation	Within- and between-run performance at relevant levels

chromatographic assays, important considerations include selectivity, calibration curve, accuracy and precision, carryover, dilution integrity where relevant, matrix effect, recovery, stability, and study-sample analysis. The exact studies required depend on the analyte, matrix, method, and applicable regulatory context.

A common conceptual error is to combine the terminology of pharmaceutical analytical validation with bioanalytical validation without clarifying the use case. For example, a formulation assay may focus on assay accuracy, precision, specificity, linearity, and robustness. A plasma assay may additionally need extensive matrix-effect testing, assessment of endogenous interference, stability under sample-handling conditions, carryover after high-concentration samples, and incurred-sample reanalysis where applicable. The appropriate regulatory framework should be named explicitly, and the validation protocol should demonstrate how each relevant risk is controlled.

relevant quantitative performance should be confirmed in the receiving laboratory [31].

AQbD can support lifecycle management when development data identify which parameters are critical and which can vary without meaningful impact. However, a broad design space should not be claimed if the evidence only supports a single set-point. A transparent control strategy is preferable to unsupported claims of robustness. Where a change could affect selectivity or matrix effect, a scientifically justified bridging study may be necessary. The scope of revalidation should be proportional to the risk and documented in accordance with the applicable quality system [32].

Matrix effect and recovery	Co-extracted components and extraction variability can alter response	Multiple matrix lots; separate recovery and matrix-effect evaluation
Carryover	High samples can contaminate subsequent low samples	Blank after high-concentration sample; predefined response
Stability	Handling and storage can change measured concentration	Bench-top, freeze-thaw, long-term and processed-sample studies as applicable
Dilution integrity	Samples above calibration range may need dilution	Validation of dilution procedure when anticipated
Lifecycle / transfer	Instrument, column and laboratory	System suitability, change assessment, bridging and

	changes can alter performance	documented monitoring
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8. Green analytical chemistry and the meaning of a “greener” method

8.1 Where environmental burdens arise

The environmental footprint of liquid chromatography is distributed across the entire analytical workflow. It includes sample collection materials, extraction solvents, buffers, disposable cartridges, filters, vials, tubing, mobile-phase preparation, column washing, instrument energy use, temperature control, waste treatment, and repeat analyses. The solvent volume consumed per injection depends on flow rate, total cycle time, gradient profile, wash steps, equilibration, and the frequency of blanks or quality-control injections. A method with a short nominal run time may still have a high solvent burden if it operates at high flow or requires extensive re-equilibration.

GAC therefore favours prevention and reduction rather than cosmetic substitution. Reducing sample and reagent volumes, minimizing unnecessary steps, selecting lower-hazard solvents where compatible, improving extraction efficiency, optimizing batch scheduling, and reducing repeat analysis can all contribute. Nevertheless, environmental choices must remain constrained by analytical fitness. If a solvent change worsens peak shape, reduces sensitivity, or increases run time enough to offset the volume reduction, the overall method may not be more sustainable. A valid comparison must consider complete workflows at equivalent performance [33].

8.2 Metrics: NEMI, Eco-Scale, GAPI, AGREE and AGREEprep

Several metrics have been developed because “greenness” is multidimensional. NEMI uses a relatively simple pictorial assessment of selected hazards and waste characteristics. Analytical Eco-Scale applies penalty points for factors such as hazardous reagents, energy, occupational risk, and waste. GAPI presents a pictorial profile across multiple stages of the analytical procedure. AGREE translates the 12 principles of GAC into a normalized score and visual representation [6-9]. AGREEprep focuses on sample-preparation steps, an important addition when extraction contributes substantially to the overall environmental burden [9]. The choice of metric should be based on the question being asked and the data available.

A broad comparison of 16 greenness metrics notes that the tools differ in criteria, output type, advantages, and limitations [6]. A separate review of green analytical chemistry metrics discusses the role of AGREE, GAPI, Analytical Eco-Scale, and other approaches in evaluating analytical procedures [7]. These reviews support use of metrics as structured decision aids, not as universal measures of environmental impact. Metrics may differ in how they treat energy, hazards, waste, sample preparation, and the analytical procedure's applicability.

A method may score well in one tool but less favourably in another because their system boundaries and weightings are different [34].

Greenness reporting should therefore include the metric name and version, the inputs used, the workflow stages included, and the assumptions made. Authors should provide raw values-such as solvent identity and volume, sample volume, extraction consumables, analysis and equilibration time, and waste categories-alongside the pictogram or score. This enables readers to understand why a score was assigned and to reproduce the comparison. Scores should not be compared directly across different tools unless a validated mapping exists.

8.3 Solvent substitution and chromatographic trade-offs

Replacing acetonitrile or methanol with another solvent can reduce some hazards, but solvent selection is a chromatographic decision as well as an environmental one. Viscosity, elution strength, UV cut-off, miscibility, pressure, analyte solubility, selectivity, and detector compatibility may all change. Ethanol, for example, may be attractive in some workflows but can increase viscosity and backpressure under certain conditions. Water-rich mobile phases can reduce organic consumption but may alter retention or require longer runs. The relevant comparison should quantify the complete method at comparable accuracy, precision, sensitivity, throughput, and robustness [35].

Solvent reduction can also be achieved by optimizing flow rate, column dimensions, gradient volume, injection volume, and equilibration. Smaller columns and lower flow may reduce solvent use, but the effects on extra-column dispersion, injection compatibility, pressure, and peak capacity must be assessed. Shorter gradients may improve throughput but can compromise separation of structurally similar compounds or allow matrix components to accumulate. The sustainability target should be defined alongside mandatory analytical criteria, not optimized independently [36].

8.4 Greenness and analytical applicability are separate dimensions

A greenness score does not measure clinical utility. A method that uses a small amount of a low-hazard solvent but cannot quantify an analyte near the clinically relevant concentration is not a suitable replacement for a more resource-intensive method. Conversely, a highly sensitive LC-MS/MS assay may be justified for PK studies even if its energy use and instrument costs are higher than those of UV detection. The decision should first exclude methods that fail the ATP and then compare environmental and operational burdens among methods that meet the required performance. This sequential approach avoids allowing a favourable greenness score to compensate for inadequate analytical quality [37].

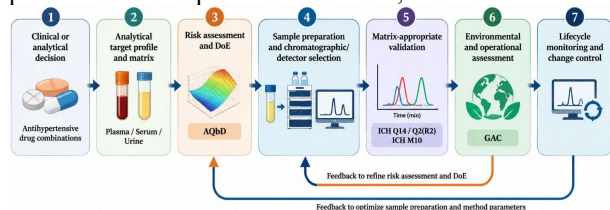
Table 4. Greenness tools interpreted according to their purpose and system boundaries [6-9].

Metric / approach	Typical contribution	Strength	Limitation to acknowledge
NEMI	Pictorial screen of selected hazards and waste criteria	Simple to communicate	Limited resolution; not a full lifecycle assessment
Analytical Eco-Scale	Penalty-point approach across selected resource and hazard factors	Makes penalties visible	Outcome depends on scoring assumptions and input completeness
GAPI	Pictorial profile across analytical	Highlights stage-specific burdens	Can be difficult to compare numerically

9. Integrating AQbD, validation and GAC into one decision framework

AQbD and GAC are often applied as separate exercises: a method is optimized and validated first, then a greenness score is calculated after development. This sequence can miss opportunities to reduce environmental burden during the design stage. An integrated approach treats analytical performance and sustainability as parallel objectives, while retaining minimum performance criteria as non-negotiable constraints. For example, DoE can evaluate mobile-phase composition, flow rate, gradient duration, extraction solvent volume, and sample volume against responses that include resolution, accuracy, matrix effect, run time, and solvent consumption. The design should not allow a low solvent volume to compensate mathematically for failure to meet a critical selectivity or precision requirement [38].

The proposed decision framework has seven linked steps. First, define the intended clinical, pharmacokinetic, or quality-control decision. Second, specify the ATP, including matrix, analytes, concentration range, and performance requirements. Third, select candidate



10. Critical comparison of representative evidence

Table 5 summarizes selected evidence that directly informs the review's central framework. These sources differ in purpose and should not be interpreted as a single comparative experiment. The 21-drug serum assay

	workflow stages		vary across methods
AGREE	Score based on the 12 principles of GAC	Structured, normalized visual summary	Score depends on inputs, weighting, and boundaries
AGREEprep	Greenness assessment focused on sample preparation	Draws attention to extraction burden	Does not by itself characterize the entire analytical lifecycle
Life-cycle assessment (where feasible)	Broader assessment of defined environmental impacts	Potentially more comprehensive	Data-intensive; results depend on system boundaries and inventory quality

sample-preparation and chromatographic technologies based on analyte properties and matrix risks. Fourth, use risk assessment and structured experiments to identify critical parameters and acceptable operating ranges. Fifth, validate the method under the guidance appropriate to the intended use. Sixth, evaluate environmental and operational burdens using transparent metrics and raw workflow data. Seventh, implement lifecycle monitoring, change control, and periodic reassessment when instruments, columns, reagents, or workflows change. This framework is intentionally sequential in its decision logic but iterative in practice. If the greenness assessment identifies a high burden from extraction, sample preparation can be redesigned and the relevant performance attributes re-tested. If a greener mobile phase changes retention or ionization, the chromatographic method must be re-optimized and the validation impact assessed. If lifecycle monitoring shows that a critical parameter is drifting, the risk assessment and control strategy should be revisited. The framework therefore creates a feedback loop rather than a one-time optimization exercise [39].

Figure 1. Integrated decision pathway linking intended use, AQbD, validation, greenness assessment and lifecycle control.

demonstrates feasibility of a broad TDM panel; the nebivolol plasma assay demonstrates targeted quantitative bioanalysis; ICH Q14, Q2(R2), and M10 establish different but complementary regulatory expectations; and GAC metric reviews show that

greenness can be evaluated through multiple non-equivalent tools [40].

Table 5. Evidence of study design, matrix, intended use and scope.

Source	Evidence type	What it supports	What it does not establish
Gundersen et al. [1]	Primary bioanalytical study	UHPLC-MS/MS quantification of 21 antihypertensive drugs in serum for TDM	Universal suitability for every drug, matrix, or clinical decision
Nandani et al. [2]	Primary bioanalytical study	Targeted LC-MS/MS quantification of nebivolol in human plasma	Performance of a broad multi-analyte panel
ICH Q14 [3]	Regulatory guideline	Science- and risk-based analytical procedure development and lifecycle concepts	A substitute for bioanalytical validation guidance in all settings

10.1 What can be compared fairly?

A fair comparison of multicomponent methods should first establish whether the methods address the same analytical question. Relevant descriptors include analyte panel, matrix, extraction, detector, calibration range, LLOQ, sample volume, validation design, and intended decision. Run time and solvent use should be calculated across the full cycle, including equilibration and cleaning. A method with a broader panel may be more efficient per analyte but may have a longer total run; a targeted method may provide better sensitivity for one analyte but cannot replace a panel without additional assays. Cross-study comparison should remain qualitative when the underlying conditions are not comparable [41].

Where environmental performance is reported, the raw inventory should accompany the metric score. This includes the volume and hazard profile of solvents, consumables, sample volume, waste handling, and energy assumptions. If one study reports AGREE and another reports GAPI, their scores should not be placed in a common ranking without a justified conversion. Similarly, a method's reported recovery cannot be compared directly with another method's recovery unless the matrix, extraction design, analyte, and calculation are sufficiently similar. These constraints do not prevent critical synthesis; they define what can be concluded responsibly [42].

ICH Q2(R2) [4]	Regulatory guideline	Validation principles for analytical procedures within its scope	A complete bioanalytical validation plan for all biological matrices
ICH M10 [5]	Regulatory guideline	Validation and study-sample analysis for bioanalytical assays	Clinical interpretation of concentrations without contextual evidence
Shi et al. [6]	Review of greenness metrics	Comparison of 16 analytical greenness tools	A single universal metric that fully captures lifecycle impact
Pena-Pereira et al. [8]	Metric-development paper	AGREE approach based on the 12 GAC principles	Analytical performance or clinical utility by itself

11. Challenges and limitations

The evidence base is heterogeneous. Primary studies differ in analyte scope, biological matrix, instrumentation, extraction method, calibration strategy, and validation design. A broad TDM panel and a single-analyte PK assay cannot be ranked by run time or sensitivity alone. Likewise, a method developed for a synthetic mixture or finished product cannot be treated as a validated bioanalytical procedure. The literature examples included here demonstrate important principles but do not constitute an exhaustive systematic inventory of all antihypertensive methods. A systematic review would be required to quantify the frequency of particular technologies, validation gaps, or sustainability practices across the field.

AQbD terminology is also used inconsistently. Some studies report a DoE and call the method QbD-based, while providing limited evidence for risk assessment, model verification, operating ranges, or lifecycle control. A statistical optimum is not automatically a robust operating region. To substantiate an AQbD claim, reports should explain how the ATP was defined, which parameters were considered critical, why factors and responses were selected, how model adequacy was assessed, and how the final control strategy was established [43].

Greenness metrics have their own limitations. The available tools differ in input requirements, weighting,

output format, and system boundaries [44]. They may not include the full burden of instrument manufacture, laboratory infrastructure, solvent production, or waste treatment. A score can also obscure trade-offs among hazard, waste, energy, and performance. Consequently, no single metric should be treated as a definitive environmental verdict. Transparent reporting of raw workflow data is necessary for meaningful comparison. Regulatory frameworks must also be applied accurately. ICH Q14 and Q2(R2) concern analytical procedure development and validation within their defined scope, while ICH M10 addresses bioanalytical methods and study-sample analysis [3-5]. A manuscript that uses these guidelines interchangeably risks misleading readers. Finally, analytical measurement does not independently prove adherence, therapeutic efficacy, or clinical benefit. Drug concentrations depend on dose timing, absorption, metabolism, elimination, co-medications, and sampling conditions. Clinical interpretation requires context beyond the chromatographic result [45].

12. Future perspectives and research roadmap

12.1 Reporting And Transparency

Future method reports should state the intended use, biological matrix, analyte panel, expected concentration range, and decision supported by the measurement. They should provide sufficient detail to reproduce sample preparation, chromatography, detection, calibration, quality-control preparation, and validation. For greenness, authors should report raw solvent volumes, solvent identities, sample volume, extraction consumables, total cycle time, equilibration, and waste categories in addition to a named metric. This would allow readers to distinguish a genuinely lower-burden workflow from one that merely scores favourably under a narrow boundary [46].

12.2 Integrated experiments

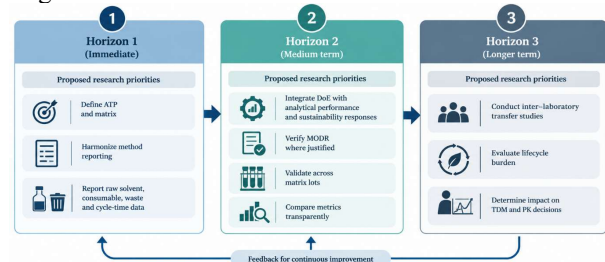
AQbD and GAC should be combined at the planning stage. DoE can be used to assess the effects of mobile-

phase composition, flow, gradient duration, extraction conditions, and sample volume on analytical performance and environmental indicators. Mandatory performance criteria should act as constraints, and the remaining feasible solutions can be compared as a trade-off or Pareto set. This is more informative than collapsing all outcomes into one desirability score with undocumented weights. Confirmatory studies should assess whether the proposed method remains suitable across multiple matrix lots and realistic operating conditions [47].

12.3 Longer-term priorities: transfer and clinical utility

Multi-laboratory studies are needed to test whether methods transfer across instruments, columns, analysts, and sample sources without unacceptable changes in performance. For TDM, research should evaluate whether multi-analyte panels improve turnaround, interpretation, or resource use relative to separate assays. For PK studies, the focus should remain on reliable concentration-time data, incurred-sample performance, and reproducibility across sites. For adherence assessment, results should be interpreted with dose timing and clinical context; a single measured concentration should not be treated as definitive proof of long-term adherence [48].

Future studies should also examine the real resource use of repeat analysis, failed quality controls, column replacement, sample storage, and waste treatment. A workflow that minimizes solvent per injection but requires frequent repeat runs may not reduce total burden. Conversely, an assay with a higher instrument energy demand may be justified if it substantially reduces sample volume or consolidates several measurements into one validated run. These are empirical questions that require transparent inventories rather than assumptions based on platform labels [49] (Figure 2).



13. Conclusion

Multicomponent antihypertensive analysis should be designed around the analytical decision and biological matrix rather than around the maximum number of analytes that can be placed in a single chromatographic run. Published studies demonstrate the feasibility of a broad UHPLC-MS/MS serum panel for TDM and targeted LC-MS/MS measurement of nebivolol in plasma [1,2]. Their results are informative within their respective scopes but do not establish universal transferability. AQbD provides a structured pathway from intended use

and risk assessment to experimentation, operating ranges, validation, and lifecycle control. GAC adds the requirement to reduce hazardous reagents, solvent, waste, and energy without compromising analytical fitness. ICH Q14 and Q2(R2) support science-based development and validation of analytical procedures, while ICH M10 is directly relevant to bioanalytical validation and study-sample analysis [3-5]. The principal research priority is to integrate these dimensions in transparent, matrix-specific studies that report both analytical performance and environmental inventory.

Figure 2. Research roadmap for multicomponent bioanalysis.

Such work would provide a stronger basis for choosing methods that are robust, transferable, and resource-

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