Journal of Medicinal Chemistry

pubs.acs.org/jmc

Article

Development and Validation of a UHPLC-MS/MS Method for the Simultaneous Quantification of Candesartan and Bisoprolol Together with Other 16 Antihypertensive Drugs in Plasma Samples

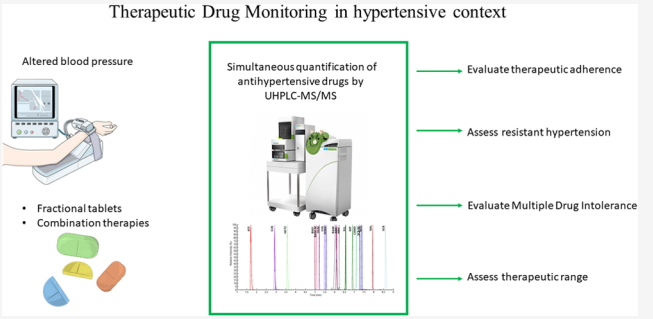
Alice Palermiti,*[,⊥] Amedeo De Nicolò,[⊥] Alessandra Manca, Miriam Antonucci, Jacopo Mula, Martina Billi, Jessica Cusato, Alessandro Carta, Marco Pappaccogli, Lara Ponsa, Chiara Fasano, Francesco Giuseppe De Rosa, Franco Rabbia, Franco Veglio,[⊥] and Antonio D'Avolio[⊥]

Cite This: J. Med. Chem. 2024, 67, 22124−22133

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Antihypertensive pharmacological therapy is often characterized by a coadministration of different classes of drugs. Therefore, analytical methods allowing the simultaneous quantification of many drugs are needed for therapeutic drug monitoring (TDM) purposes in this context. In particular, TDM represents a useful tool to discriminate poor adherence from real cases of resistant hypertension. For this reason, the aim of this study is to validate, following the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) guidelines, an ultrahigh-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) method for the simultaneous quantification of 18 antihypertensive drugs in human plasma. A LX-50 coupled with a QSight 220 UHPLC-MS/MS system with electrospray ionization and multiple reaction monitoring mode was used, after a binary gradient separation (13 min) on a reverse-phase Acquity UPLC HSS T3 [1.8 μm, 2.1 mm × 150 mm] column. Method validation showed a stable and acceptable matrix effect, recovery, high accuracy, and precision, assessing the eligibility of this method for routine use in the clinical context.



1. INTRODUCTION

Among the major causes of death in the world, cardiovascular diseases are preponderant factors.[1−3] In particular, hypertension represents the main risk factor for coronary syndrome, myocardial infarction, heart failure, and peripheral arterial disease, as well as being one of the main causes of chronic kidney disease.[4,5] Therapeutic approaches to treat hypertension can be pharmacological[5,6] or nonpharmacological.[7−11]

In 85% of the population with hypertension, drug therapy in combination with a correct lifestyle works and blood pressure is restored to normal values.[12] Conversely, in a subset of patients, suboptimal control of blood pressure persists due to different factors such as failure in the modification of lifestyle and diet, secondary forms of hypertension, treatment-refractory hypertension, and adverse drug reaction onset leading to poor therapeutic adherence.[13−17]

In this context, therapeutic drug monitoring (TDM) is an example of clinical practice to optimize the pharmacological approach. TDM comprises the determination of plasma concentrations of a drug and possible posological adjustments based on these findings.[18] The fundamental concept is represented by the presence of both efficacy and toxicity being concentration-dependent, so that an optimal range of drug concentrations is identified with high probability of therapeutic efficacy and low probability to observe toxic effects.[18,19]

Unfortunately, to date, there are no generally acknowledged therapeutic ranges for antihypertensive drugs and therefore the use of TDM is mostly limited to the evaluation of therapeutic adherence, allowing to identify not only poor adherence but also pharmacokinetic interactions or issues in drug absorption, distribution, metabolism, and elimination (ADME).[20]

One of the main advantages of using TDM to check adherence is the prevention of overly invasive therapeutic procedures such as renal denervation (RDN) or baroreceptor implantation without real indication in patients who could simply benefit from better counseling with their physician and consequent adjustments in the treatment to solve potential issues with treatment-related adverse events. The dimension of

Received: August 27, 2024
Revised: November 26, 2024
Accepted: November 27, 2024
Published: December 4, 2024

https://doi.org/10.1021/acs.jmedchem.4c02045
J. Med. Chem. 2024, 67, 22124−22133

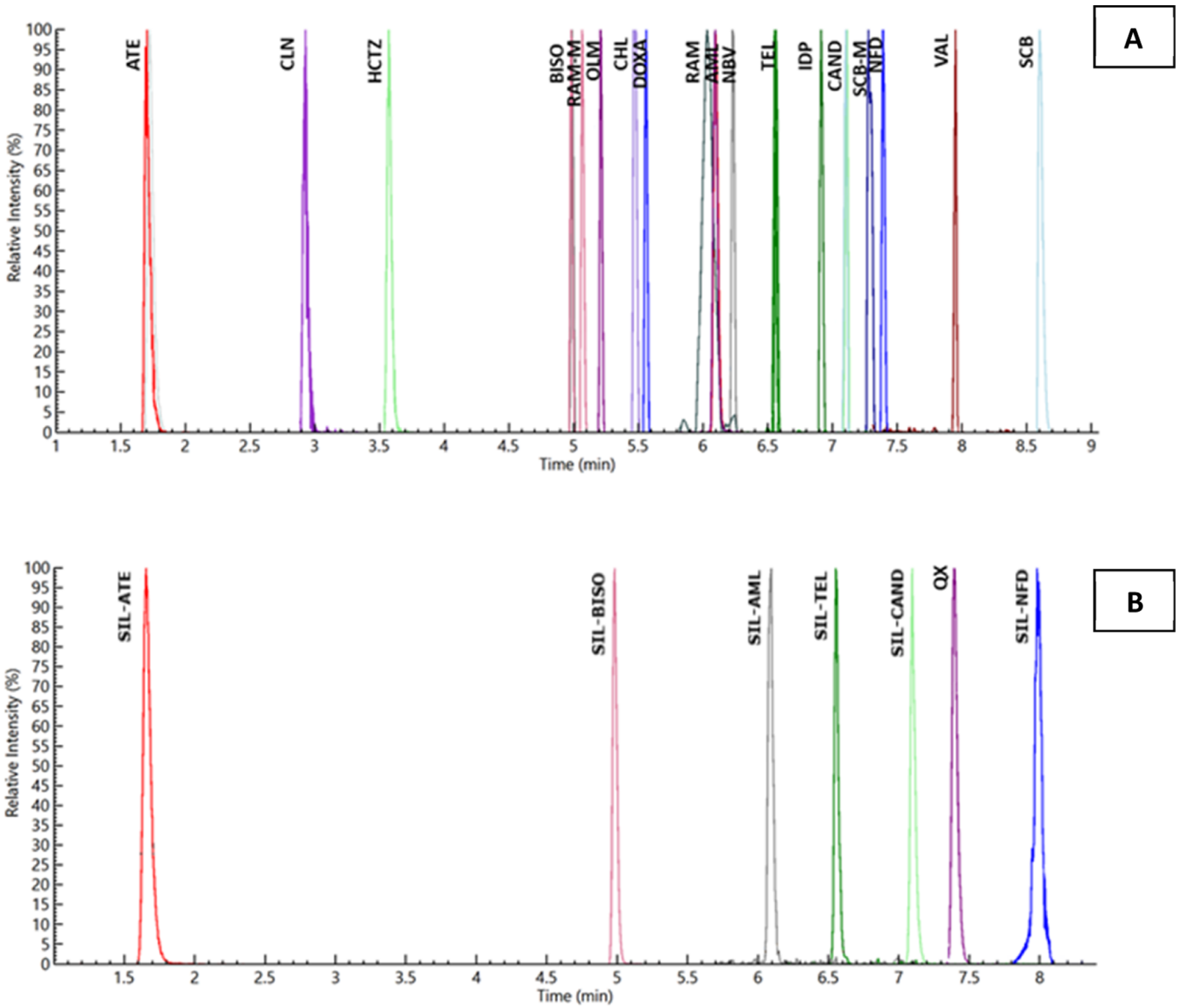


Figure 1. Chromatographic run and analyte separation after the injection of the highest curve calibrator (A) and the consequent blank plasma injection (B)*n*. ATE-D, [$^{2}H_{7}$]-ATE; AML-D, [$^{2}H_{4}$]-AML; NFD-D, [$^{13}C_{8}$]-NFD; TEL-D, [^{13}C, $2H_{3}$]-TEL; CAND-D, [$^{2}H_{4}$]-CAND; and BISO-D, [$^{2}H_{7}$]-BISO.

this problem can be extremely relevant; in recent works by our group, systematic use of TDM of 10 drugs in a cohort of patients with resistant hypertension showed that about 50% of them had poor adherence, explaining their lack of control in blood pressure.[15,16] This poor adherence could be partially explained by high pill burden (pill fatigue), patient's distrust in the pharmacological therapy, side effects due to comorbidities, or the multiple drug intolerance phenomenon. For this reason, close monitoring of blood pressure together with the practice of TDM could represent a complete procedure in clinical practice to avoid invasive procedures such as RDN. Therefore, considering that the majority of patients who could benefit from TDM receive a combination therapy, an analytical method for the simultaneous quantification of several drugs in matrices where the drugs should be present would be extremely useful.

However, there are a few works in literature validating drug quantification methods in plasma, urine, or saliva,[21−24] with a large panel of antihypertensive drugs, including those widely used in clinical practice, often in combination (e.g., therapy of controlled-release nifedipine plus candesartan),[25] creating the need to quantify as many drugs as possible simultaneously, especially in those patients taking multiple drugs. For these reasons, therefore, the aim of this study was to update and validate a UHPLC-MS/MS method, following the Bioanalytical Method Validation Guidance for Industry from the FDA and the EMA,[26,27] for the simultaneous quantification of the following 18 antihypertensive drugs in human plasma: atenolol (ATE), nebivolol (NBV), clonidine (CLN), olmesartan (OLM), telmisartan (TEL), valsartan (VAL), amlodipine (AML), nifedipine (NFD), doxazosin (DOX), chlorthalidone (CHL), hydrochlorothiazide (HCTZ), indapamide (IDP), sacubitril (SCB) and its metabolite sacubitrilat (SCB-M), and ramipril (RAM) and its metabolite ramiprilat (RAM-M), updating the method with the introduction of bisoprolol (BISO) and candesartan (CAND).

https://doi.org/10.1021/acs.jmedchem.4c02045
J. Med. Chem. 2024, 67, 22124−22133

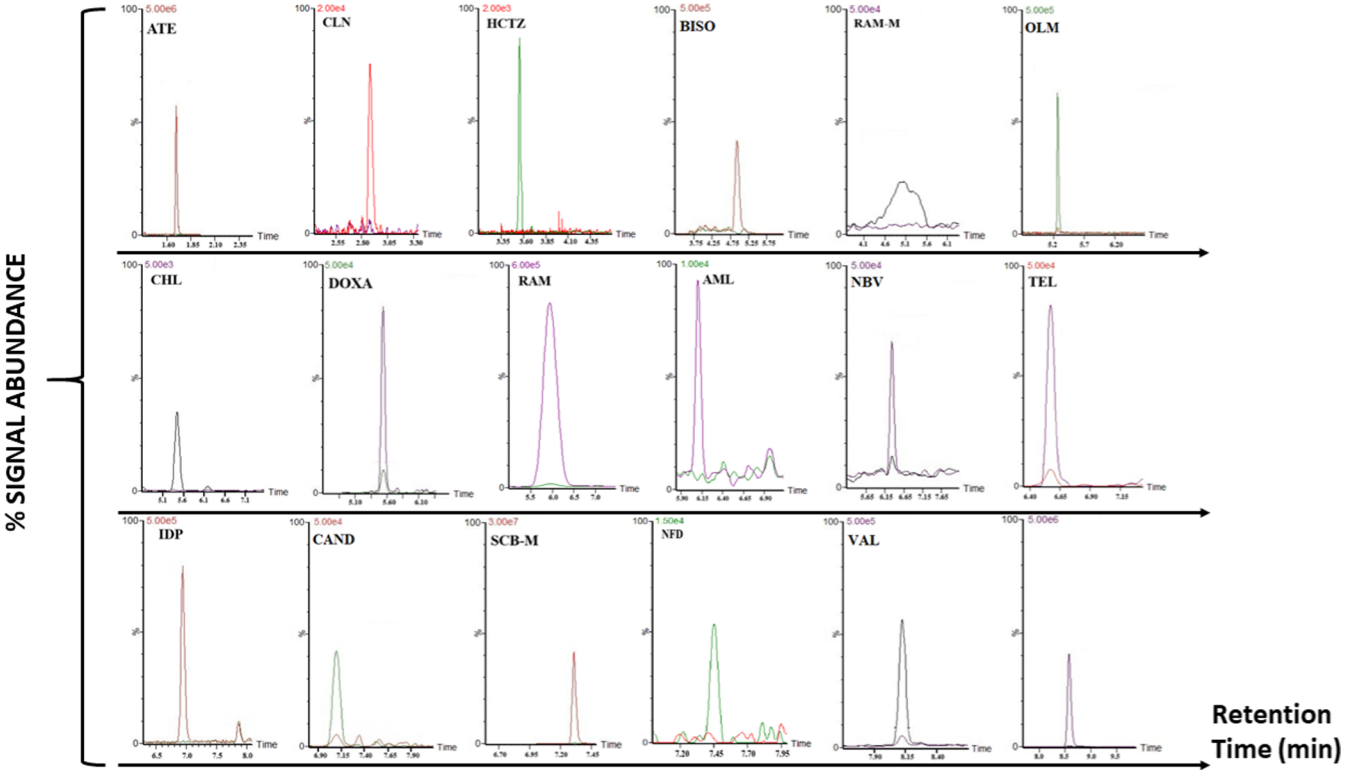


Figure 2. Lower limit of quantification (LLOQ) overlaid to the blank sample injected after high-concentration standard chromatograms, expressed in percent signal abundance versus retention time, for each analyte.

2. RESULTS

Previous work by our research group dealt with the validation in plasma of the method of 16 molecules useful in clinical practice;[22] this method included 18 molecules instead, and the validation results are consistent with the previous one. For this reason, only the results relating to the new drugs added to the method will be highlighted: BISO and CAND.

2.1. Specificity and Selectivity. All of the analytes were retained following a chromatographic separation based on their mass, pK_a, and polarity characteristics. Mean retention times (RT, Figure 1A) for the considered analytes were reported as follows: 1.69 min (±0.05) for ATE, 2.90 min (±0.05) for CLN, 3.55 min (±0.05) for HCTZ, 4.98 min (±0.05) for BISO, 5.10 min (±0.05) for RAM-M, 5.20 min (±0.05) for OLM, 5.50 min (±0.05) for CHL, 5.54 min (±0.05) for DOXA, 6.02 min (±0.05) for RAM, 6.08 min (±0.05) for AML, 6.30 min (±0.05) for NBV, 6.55 min (±0.05) for TEL, 6.91 min (±0.05) for IDP, 7.10 min (±0.05) for CAND, 7.28 min (±0.05) for SCB-M, 7.44 min (±0.05) for NFD, 8.15 min (±0.05) for VAL, and 8.60 min (±0.05) for SCB. Deuterated analytes reported in Figure 1B were considered to normalize the validation results as the internal standard (IS).

Each analyte quantified through the method had its corresponding IS, chosen based on the chemical composition, pK_a, and polarity, yielding similar RT during the chromatographic run; for these reasons, stable isotope-linked molecules (SIL-IS) have been used when available and economically affordable. As follows, [$^2H_4$]-AML was the IS of AML and DOXA, while [$^2H_7$]-ATE was used both for ATE and CLN; [$^{13}C_8$]-NFD, [$^2H_4$]-CAND, and [$^2H_7$]-BISO were used to normalize NFD, BISO, and CAND, respectively; [^{13}C, $^2H_3$]-TEL for TEL, OLM, NBV, CHL, and IDP; and finally, 6,7-dimethyl- 2,3-di(2-pyridyl)quinoxaline (QX) for the remaining compounds.

Moreover, as shown in Figure 2, it is possible to observe the overlaid chromatogram representing the % of the signal intensity of the blank sample injected after the highest calibration standard and the LLOQ (Calibrator 1) for each analyte present in the method; these results are consistent with those of the previous validated method.[15] The blank plasma sample presented no interfering peaks at the corresponding analyte RT.

2.2. Accuracy, Precision, Linearity, and Limit of Quantification. Accuracy values for BISO and CAND at each different concentration were as follows: BISO accuracy values were 99.7, 96.61, and 96.87% concerning high quality control (QC-H), medium quality control (QC-M), and low quality control (QC-L) levels, respectively, while considering CAND, accuracy values were 100.7, 101.7, and 91.28% for each concentration level.

Also, intraday imprecision was analyzed for each concentration level; considering BISO, the intraday imprecision calculated had an RSD of 2.8% for QC-H, 0.5% for QC-M, and 1.9% for QC-L; likewise, CAND showed an RSD of 2.8% (QC-H), 2.2% (QC-M), and (QC-L) 7.7%.

Finally, interday imprecision was evaluated, and BISO showed an RSD of 2.38% for QC-H, 1.42% for QC-M, and 3.84% for QC-L, while CAND showed an RSD of 3.43% (QC-H), 2.14% (QC-M), and 7.98% (QC-L).

All of these parameters fitted the FDA and the EMA guidelines.[26−28]

The complete table of accuracy and precision for all of the antihypertensive compounds is reported in the Supporting Information (Table S1).

https://doi.org/10.1021/acs.jmedchem.4c02045
J. Med. Chem. 2024, 67, 22124−22133

Moreover, curve calibration parameters for BISO and CAND were calculated and are reported in Table 1.

Table 1. Curve Calibration Parameter for Candesartan and Bisoprolol UHPLC/MS-MS Analysis

plasma validation parameter	candesartan	bisoprolol
linearity range (ng/mL)	156−400	097−250
correlation factor (R^2)	099902	099962
slope (m)	0000321584	003955
intercept (q)	0000625786	00537
limit of detection ng/mL (LOD)	047	03
lower limit of quantification ng/mL (LLOQ)	156	097

The lower limit of quantification (LLOQ) values were 1.56 ng/mL for CAND and 0.97 ng/mL for BISO.

Concerning the limit of detection (LOD) parameter, a signal-to-noise ratio higher than 3 was observed by dilution of the LLOQ until the concentrations of 0.37 ng/mL for CAND and 0.30 ng/mL for BISO were obtained.

Calibration curve-fitted linear regression models: a weighting factor of 1/X was used to ensure high accuracy at low concentrations. Regression coefficients (R^2) of calibration curves were all above 0.996 (Table 1).

2.3. Recovery and Matrix Effect. To evaluate the drug recovery and matrix effect, 6 different analyses on different plasma lots were performed at 3 different concentrations for each drug (QC-H, QC-M, QC-L). Table 2 lists the recovery data for each drug together with the IS mean recovery and IS-normalized matrix effect RSD. The recovery and matrix effect data for the other molecules contained within the method are listed in Table S1.

2.4. Freeze and Thaw Analyses. BISO and CAND stability after freezing and thawing events was conducted considering each QC level (QC-H, QC-M, QC-L). The results are reported in Figure 3A for BISO and in Figure 3B for CAND.

2.5. Long-Term Stability. The results obtained after long-term stability at −20 °C are reported in Figure 4. After 3 months, the percent deviations were 2.20% (QC-H) and 10.94% (QC-L) concerning CAND (Figure 4A) and 0.11% (QC-H) and 10.98% (QC-L) concerning BISO (Figure 4B).

2.6. Clinical Application and Incurred Sample Reanalysis. Nine patients were tested for method evaluation in real-life clinical practice; six patients were administered BISO and three were administered CAND.

All patients were treated with different drug posologies: concerning BISO, two patients with a dose of 1.25 mg, two with 2.5 mg, and two with 5 mg; and two CAND-treated patients with 16 mg and one with 8 mg, respectively.

Each plasma sample has been analyzed through the method for 3 times, after freezing and thawing cycles.

Plasma drug concentrations are reported in Figure 5, together with the stability after freezing and thawing cycles.

Unfortunately, one patient treated with BISO was excluded by the analysis since the observed concentration was below the LLOQ.

3. DISCUSSION AND CONCLUSIONS

The increase in the incidence of polytreated patients exposes the need in clinical practice to validate methods, in the context of TDM, which are able to guarantee the quantification of multiple drugs simultaneously in order to guarantee adequate patient management.

However, today, there are numerous drugs on the pharmaceutical market for the treatment of hypertension, making this goal particularly complicated.[6]

The novelty of this work consists in allowing the simultaneous quantification of 18 molecules, which contains the drugs mainly used in clinical practice; in particular, both CAND and BISO are often administered with HCTZ in combination therapy or NFD.[29−31]

The extraction method, despite being based on a simple protein precipitation, was optimized to obtain a really clear supernatant and a stable recovery of all of the analytes. The relatively long chromatographic run (13 min) allows an adequate chromatographic resolution, avoiding excessive coelution of different compounds, thus reducing the probability to experience a significant matrix effect, interaction with possible contaminants from plasma, or cross-talk from other analytes (Figure 1A). Moreover, the method does not suffer from the carry-over phenomenon, as no significant peaks were detected in a blank sample after the run of a sample that contained all of the drugs (Figure 2).

All of the accuracy and precision parameters of the method fitted the EMA and the FDA guidelines at all of the tested concentration levels.[26−28] Calibration parameters of CAND and BISO were optimal, having a determination coefficient >0.996 (Table 2).

The proposed calibration ranges have been chosen on the basis of the expected drug concentrations at both peak and trough levels and in order to cover the majority of clinically relevant scenarios, with different treatment posology.[32−34]

Deepening in the context of recovery and matrix effect analysis, it is possible to highlight how CAND showed a mean percent recovery above 120% in both QC-M and QC-L concentrations; nevertheless, this issue was adequately compensated by its IS (Table 2), as indicated by the IS-n REC values, which appeared nearer to 100% and were more reproducible, resulting in compliance with the EMA and the FDA guideline requirements.[27,28]

The calibrators and QCs were stored at −20 °C, and to evaluate BISO and CAND stability, freezing and thawing

Table 2. Summary of Validation Parameters

		recovery mean % (RSD)	matrix effect mean % (RSD)	extraction efficiency mean % (RSD)	IS-n recovery mean % (RSD)	IS-n matrix effect mean % (RSD)	IS-n extraction efficiency mean % (RSD)
CAND	H	98.2 (11.2)	96.1 (18.8)	102.2 (8.1)	80.5 (3.3)	88.7 (0.7)	86.8 (2.4)
	M	139.8 (10.6)	118.6 (0.6)	117.9 (10.4)	112.5 (2.2)	94.4 (2.0)	119.2 (3.1)
	L	146.2 (9.1)	119 (7.2)	123.0 (12.6)	102.8 (4.6)	103.5 (4.6)	83.7 (0.2)
BISO	H	106.2 (3.0)	107.1 (2.6)	99.1 (2.6)	81.5 (7.7)	98.2 (1.2)	82.9 (7.1)
	M	10.4 (1.8)	94.8 (4.2)	101.9 (2.2)	102.4 (1.55)	103.3 (0.7)	99.2 (2.2)
	L	123.1 (1.2)	110.2 (1.9)	114.3 (2.7)	109.5 (5.8)	104.9 (1.7)	104.1 (3.9)

https://doi.org/10.1021/acs.jmedchem.4c02045
J. Med. Chem. 2024, 67, 22124−22133

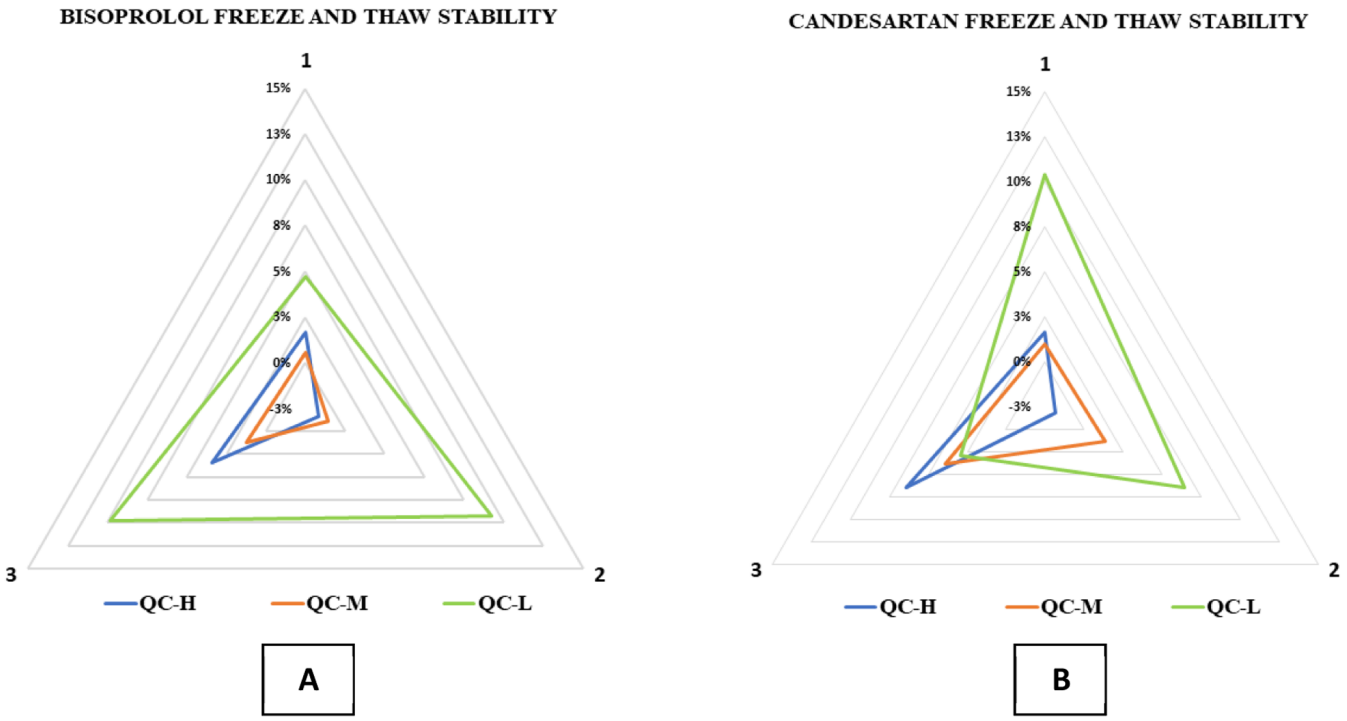


Figure 3. Freeze and thaw stability analysis of bisoprolol (A) and candesartan (B).

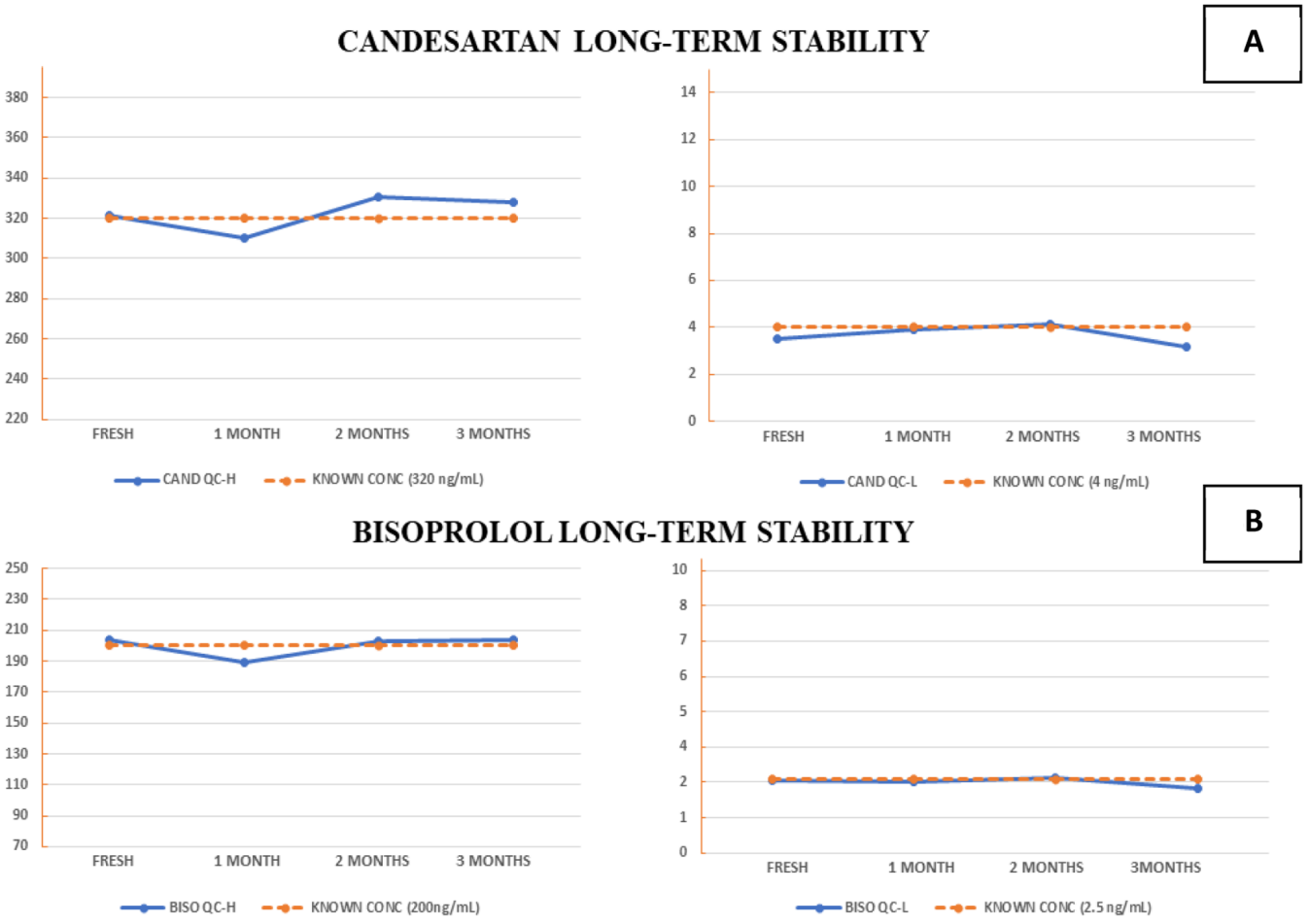


Figure 4. Long-term stability for candesartan (A) and bisoprolol (B) at −20 °C for high (QC-H) and low (QC-L) concentrations.

https://doi.org/10.1021/acs.jmedchem.4c02045
J. Med. Chem. 2024, 67, 22124−22133

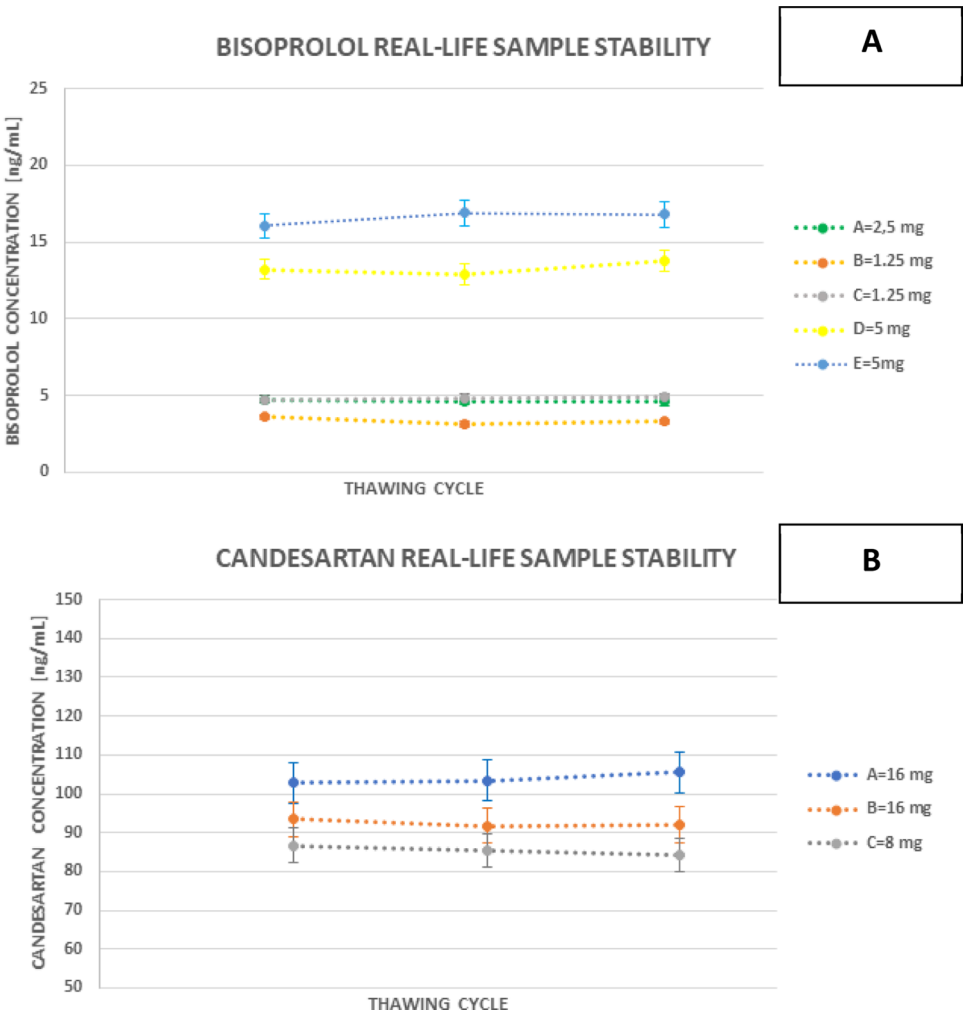


Figure 5. Bisoprolol (A) and candesartan (B) real-life test and stability analysis.

experiments together with long-term stability analyses were performed.

Figure 3, shows how both BISO and CAND QCs remain stable even after 3 cycles of freezing and thawing, reaching RSD values lower than 10%. Moreover, BISO and CAND concentrations remain stable also after 2 months of cryopreservation with a percent deviation from the default amount lower than 8% for both drugs at different ranges of concentrations (Figure 4).

Real-life samples were tested in order to complete the clinical validation; the calibration ranges proposed were congruent with the BISO and CAND plasma concentrations found in patients. After retesting each sample for 3 times, it is possible to see how each quantification did not fluctuate more than 5%, as can be seen from the error bar reported in Figure 5.

The method has been tested on real plasma samples from patients treated with BISO and CAND and, as expected, was capable of correctly quantifying target drugs within the range of measure, supporting the precision and accuracy measurements established with the QCs. The robustness and precision of the quantification method make this method suitable for drug quantification in clinical practice. Going deeper in the clinical sample analyses, it is possible to highlight how one patient treated with BISO at 2.5 mg showed a plasma concentration below the del LLOQ, making its quantification not reliable. This very low BISO amount in plasma exposure may be due to a scarce compliance, differences in drug metabolism and drug elimination, genetics, or other factors, which are yet to be investigated.[17,35−38]

Going deeper in this context, since enrolled patients were part of a study aimed to discriminate resistant hypertension from scarce adherence and from multiple drug intolerance cases, scheduling of unannounced sampling was performed during the outpatient visits with prior completion of drug adherence questionnaires. This type of enrollment, unfortunately, does not allow for selected time points near the Cmax base on the pharmacokinetic profile of these classes of drugs because the timing of sampling is limited to those reported by patients, which may or may not be accurate.

Despite this aspect, we can assess that clinical validation had a very low sample size, but all of the samples analyzed reported a high robustness and reproducibility of measurement, making this method eligible for clinical purposes.

Summarizing, all validation parameters fitted the requirements from the FDA and the EMA guidelines.[26,28]

Other than for TDM purposes, in order to evaluate therapeutic adherence[15] and/or to optimize the posology, due to the wide calibration range, this method can be useful for future pharmacokinetic studies, such as for drug−drug interactions or new formulations, since it is capable of describing the full range of concentrations retrievable during

https://doi.org/10.1021/acs.jmedchem.4c02045
J. Med. Chem. 2024, 67, 22124−22133

the 24 h (both peak and trough). Finally, this method could be useful, in the future, in order to study the pharmacokinetic–pharmacodynamic properties of combination therapies, possibly assessing their ideal therapeutic ranges in real-life clinical use.

As a future perspective, we plan to test this method on as many real samples as possible, continuing to enroll patients to complete the incurred sample reanalysis suggested by the guidelines. Moreover, a multiplexed method as the one presented in this work could be adapted and validated for the simultaneous quantification of these drugs in other less invasive and convenient matrices for the assessment of therapeutic adherence, such as saliva and urine, increasing the applicability of TDM practice in clinical centers where blood withdrawal is not possible.

4. EXPERIMENTAL SECTION

4.1. Chemicals. HPLC-grade acetonitrile (ACN) and methanol (MetOH) were purchased from VWR (Milan, Italy). HPLC-grade water (H_2O) was produced using a Milli-DI system coupled with a Synergy 185 system by Millipore (Milan, Italy). Formic acid (F.A.) and QX (purity 99.9%) were purchased from Sigma-Aldrich Corporation (Milan, Italy).

CAND (purity 97.4%), BISO hemifumarate (purity 99.4%), TEL (purity 99.9%), DOXA (purity 98.0%), ATE (purity 99.9%), NBV (purity 99.7%), HCTZ (purity 99.5%), NFD (purity 99.1%), RAM (purity 99.8%), CHL (purity 99.8%), AML (purity 99.8%), OLM (purity 99.9%), IDP (purity 99.7%), VAL (purity 99.4%), RAM-M (purity 98.0%), SCB (purity 99.4%), SCB-M (purity 99.0%), CLN (purity 98.0%) powders, and all of the isotope-labeled molecules used as internal standards (IS) ([2H4]-AML maleate, [13C8]-NFD, [13C, 2H3]-TEL, [2H4]-CAND (purity 95.0%), and [2H7]-BISO hemifumarate (purity 98.5%)) were purchased from Alsachim (Illkirch Graffenstaden, France).

All of the other powders were purchased from MedChem Express (Monmouth Junction, NJ).

All compounds are >95% pure by HPLC analysis. All powders were stored in the dark, at −20 °C according to instructions, to prevent any possible degradation.

4.2. Stock Solutions, Internal Standard, Standards, and Quality Controls. Stock solutions (1 mg/mL) were prepared as follows:

DOXA, AML, CHL, HCTZ, NFD, OLM, RAM, RAM-M, IDP, VAL, SCB, SCB-M, [$^2H_4$]-AML maleate, and QX stock solutions in a mixture of H_2O:MetOH 5:95 (v:v); [^{13}C, $^2H_3$]-TEL and TEL in dimethyl sulfoxide (DMSO):MetOH 50:50 (v:v); ATE and NBV in H_2O:MetOH 50:50 (v:v); [$^{13}C_8$]-NFD, CAND, and [$^2H_4$]-CAND in pure MetOH; CLN and BIS hemifumarate in pure H_2O; and [$^2H_7$]-BIS hemifumarate in ACN:MetOH 50:50 (v:v). All of the stock solutions were stored at −20 °C until use (<1 month).

Single aliquots of Standard 9 (STD9) and QCs were prepared by independently spiking blank plasma from healthy donors with stock solutions and then stored at −80 °C.

Calibration ranges and QC levels for all of the molecules in the method are summarized in Table S3. The IS working solution was prepared by diluting the isotope-labeled molecules and QX stock solution in a solution of H_2O:MetOH [50:50] at each analytical session, obtaining an adequate concentration inside the calibration range for each analyte.

4.3. Sample Preparation. Sample preparation consists of a protein precipitation, followed by evaporation of the precipitant solution, and all of the process is reported in Figure 6.

Briefly, 40 μL of the IS working solution was added to 200 μL of the plasma sample. After vortex-mixing for 10 s, 1 mL of ACN was added as a precipitating solution. Samples were vortex-mixed for 10 s and centrifuged at 21,000*g* at 4 °C for 10 min (with low brake) to ensure the formation of a protein pellet.

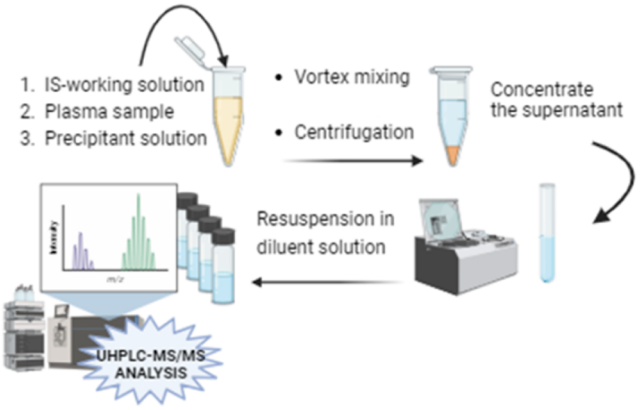


Figure 6. Sample extraction protocol created with BioRender.com.

After the centrifugation step, all of the supernatant was transferred into a vacuum concentrator at 50 °C (Labconco CentriVap Benchtop Vacuum Concentrator II). The dried samples were then resuspended with 200 μL of H_2O:ACN 90:10 (v:v) + 0.05% F.A. and transferred into glass vials. Finally, 5 μL was injected in the chromatographic system.

4.4. UHPLC-MS/MS Instruments and Chromatographic Conditions. The chromatographic system was a PerkinElmer LX-50 UHPLC system coupled with a QSight 220 Triple Quadrupole detector. The chromatographic separation was performed through an Acquity UPLC HSS T3 [1.8 μm, 2.1 × 150 mm] column (Waters, Milan, Italy), protected by a physical frit [0.2 m, 2.1 mm] (Waters, Milan, Italy) precolumn at 40 °C using a column thermostat, with a gradient of two mobile phases (Table 3): phase A (H_2O + F.A. 0.05%) and phase B (ACN + F.A. 0.05%).

Table 3. Chromatographic Gradient

time (min)	flow (mL/min)	phase A (%)	phase B (%)
0.00	0.40	90	10
1.10	0.40	90	10
9.00	0.40	25	75
9.60	0.40	5	95
10.65	0.40	90	10
11.00	0.40	90	10
13.00	0.40	90	10

The instrument was settled in positive electrospray ionization mode (ESI+) for all drugs, except for HCTZ and CHL, which was detected in negative ionization mode (ESI−). General mass settings and MRM ion traces for all of the molecules in the method are available in Table S2.

4.5. Accuracy, Precision, and Limit of Quantification. To evaluate the drug recovery and matrix effect of the method, six validation sessions were performed.

Moreover, different plasma lots from different healthy donors (obtained by "Centro Validazione Produzione Emocomponenti", O.I.R.M. Sant'Anna Hospital, Turin, Italy) were used for the preparation of the standard calibrator and quality controls (QCs) in order to ensure a good calculation of the matrix effect.

Consequently, intraday and interday precisions were evaluated performing the quantification at each validation session of 3 different QC samples at QC-H, QC-M, and QC-L concentrations.

Intraday precision was performed in 5 intraday replicates.

Interday and intraday imprecisions were expressed as %RSD at each QC level.

Integration was performed by considering peak areas for each analyte.

The LLOQ corresponds to the lower amounts of each calibration curve.

https://doi.org/10.1021/acs.jmedchem.4c02045
J. Med. Chem. 2024, 67, 22124−22133

Percentages of deviation from the nominal concentration (measure of accuracy) and relative standard deviations (measure of precision) at the concentrations considered as the LLOQ for each analyte had to be lower than 20%, as requested by the FDA and the EMA[26,28,39] guidelines.

On the other hand, the LOD has been considered as the lower dilution of the LLOQ, which yielded a signal-to-noise ratio higher than 3.

The summary of drug concentrations in standards and QC samples for each drug, together with the LOD and LLOQ values, is reported in Table S3.

4.6. Recovery. Recovery was evaluated by comparing peak areas from extracted QC-H, QC-M, and QC-L (pre-extraction-spiked) with those obtained by blank plasma extracts spiked with the same concentration of analytes (postextraction-spiked). For this reason, a neat solvent solution at the same drug concentrations as for the theoretical 100% recovery of QC-H, QC-M, and QC-L concentrations in H_2O:ACN 90:10 (v:v) + 0.05% F.A. was performed, and this solution was used to spike the blank extracts (postspike).

4.7. Matrix Effect. The matrix effect was evaluated by comparing the postspike signal QC-H, QC-M, and QC-L levels in different plasma lots with the ones from the direct injection of the same concentration of analytes in the solvent (in this case H_2O:ACN 90:10 (v:v) + 0.05% F.A.).[40]

Moreover, in order to analyze the good quality of the method, the IS-normalized matrix effect (IS-*n*ME) has been evaluated.[41]

4.8. Stock Solution Stability. Since analyte stabilities in stock solutions are already known in literature, no further experiments have been performed in this context.[20]

4.9. Freeze and Thaw Stability. To evaluate BISO and CAND stability after freezing and thawing events, the same aliquot for each concentration level considered (QC-H, QC-M, QC-L) was repeatedly analyzed for 3 times, after a new freezing cycle (24 h at −20 °C) compared with a "fresh-spiked" aliquot.

4.10. Long-Term Stability. The long-term stability for plasma QC-H and QC-L were evaluated after one, two, and three months of storage at −20 °C, considering the deviation from the expected concentration.

4.11. Incurred Sample Analysis/Reanalysis. Real samples were analyzed and reanalyzed in different runs, during the study, to rigorously validate the precision and accuracy determined by the QCs as suggested by guidelines.

The AOU Città della Salute e della Scienza di Torino committee approved the experimental procedures for the "Evaluation of the prevalence and clinical-laboratory characteristics of intolerance to single and multiple drug classes in hypertensive subjects (MDI-TO)" study (approval no. 00337/2022).

Signed informed consent was obtained before participation, during outpatient visit, prior to undergoing blood withdrawal.

■ ASSOCIATED CONTENT

Data Availability Statement

The authors declare that the data supporting the findings of this study are available in the Supporting Information files and from the corresponding author upon request.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.4c02045.

Summary of validation parameters for each analyte in the method (Table S1); general detector settings and mass transitions for each analyte (Table S2); and summary of drug concentrations in standards and quality control samples for each drug (Table S3) (PDF)

Molecular formula strings (CSV)

■ AUTHOR INFORMATION

Corresponding Author

Alice Palermiti − *Laboratory of Clinical Pharmacology and Pharmacogenetics, Department of Medical Sciences, University of Turin, 10149 Turin, Italy;* orcid.org/0000-0003-0860-280X; Email: alice.palermiti@unito.it

Authors

Amedeo De Nicolò − *Laboratory of Clinical Pharmacology and Pharmacogenetics, Department of Medical Sciences, University of Turin, 10149 Turin, Italy*

Alessandra Manca − *Laboratory of Clinical Pharmacology and Pharmacogenetics, Department of Medical Sciences, University of Turin, 10149 Turin, Italy*

Miriam Antonucci − *ASL Città di Torino, Amedeo di Savoia Hospital, 10149 Turin, Italy*

Jacopo Mula − *Laboratory of Clinical Pharmacology and Pharmacogenetics, Department of Medical Sciences, University of Turin, 10149 Turin, Italy*

Martina Billi − *Laboratory of Clinical Pharmacology and Pharmacogenetics, Department of Medical Sciences, University of Turin, 10149 Turin, Italy*

Jessica Cusato − *Laboratory of Clinical Pharmacology and Pharmacogenetics, Department of Medical Sciences, University of Turin, 10149 Turin, Italy*

Alessandro Carta − *Laboratory of Clinical Pharmacology and Pharmacogenetics, Department of Medical Sciences, University of Turin, 10149 Turin, Italy*

Marco Pappaccogli − *Division of Internal Medicine and Hypertension Unit, Department of Medical Sciences, University of Turin, 10126 Turin, Italy*

Lara Ponsa − *Division of Internal Medicine and Hypertension Unit, Department of Medical Sciences, University of Turin, 10126 Turin, Italy*

Chiara Fasano − *Division of Internal Medicine and Hypertension Unit, Department of Medical Sciences, University of Turin, 10126 Turin, Italy*

Francesco Giuseppe De Rosa − *Unit of Infectious Diseases, Department of Medical Sciences, University of Turin, 10149 Turin, Italy*

Franco Rabbia − *Division of Internal Medicine and Hypertension Unit, Department of Medical Sciences, University of Turin, 10126 Turin, Italy*

Franco Veglio − *Division of Internal Medicine and Hypertension Unit, Department of Medical Sciences, University of Turin, 10126 Turin, Italy*

Antonio D'Avolio − *Laboratory of Clinical Pharmacology and Pharmacogenetics, Department of Medical Sciences, University of Turin, 10149 Turin, Italy*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jmedchem.4c02045

Author Contributions

⊥A.P., A.D.N., F.V., and A.D.A. contributed equally to this woirk.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

ACN, acetonitrile; ADME, absorption, distribution, metabolism, and excretion/elimination; AML, amlodipine; ATE, atenolol; BISO, bisoprolol; CAND, candesartan; CHL,

https://doi.org/10.1021/acs.jmedchem.4c02045
J. Med. Chem. 2024, 67, 22124−22133

chlorthalidone; CLN, clonidine; DOX, doxazosin; EMA, European Medicines Agency; F.A., formic acid; FDA, Food and Drug Administration; H_2O, water; HCTZ, hydrochlorothiazide; IDP, indapamide; IS, internal standard; IS-nME, IS-normalized matrix; LLOQ, lower limit of quantification; LOD, limit of detection; MetOH, methanol; NBV, nebivolol; NFD, nifedipine; OLM, olmesartan; QC, quality control; QC-H, high quality control; QC-L, low quality control; QC-M, medium quality control; QX, 6,7-dimethyl-2,3-di(2-pyridyl)-quinoxaline; RAM, ramipril; RAM-M, ramiprilat; R^2, regression coefficient; RDN, renal denervation; RSD, relative standard deviation; SIL, stable isotope-linked; SCB, sacubitril; SCB-M, sacubitrilat; STD9, standard 9; TDM, therapeutic drug monitoring; TEL, telmisartan; UHPLC-MS/MS, ultrahigh-performance liquid chromatography coupled with tandem mass spectrometry; VAL, valsartan

REFERENCES

(1) Mancia, G.; Kreutz, R.; Brunström, M.; Burnier, M.; Grassi, G.; Januszewicz, A.; Muiesan, M. L.; Tsioufis, K.; Agabiti-Rosei, E.; Algharably, E. A. E.; Azizi, M.; Benetos, A.; Borghi, C.; Hitij, J. B.; Cifkova, R.; Coca, A.; Cornelissen, V.; Cruickshank, J. K.; Cunha, P. G.; Danser, A. H. J.; Pinho, R. M.; de Delles, C.; Dominiczak, A. F.; Dorobantu, M.; Doumas, M.; Fernández-Alfonso, M. S.; Halimi, J.-M.; Járai, Z.; Jelaković, B.; Jordan, J.; Kuznetsova, T.; Laurent, S.; Lovic, D.; Lurbe, E.; Mahfoud, F.; Manolis, A.; Miglinas, M.; Narkiewicz, K.; Niiranen, T.; Palatini, P.; Parati, G.; Pathak, A.; Persu, A.; Polonia, J.; Redon, J.; Sarafidis, P.; Schmieder, R.; Spronck, B.; Stabouli, S.; Stergiou, G.; Taddei, S.; Thomopoulos, C.; Tomaszewski, M.; Van de Borne, P.; Wanner, C.; Weber, T.; Williams, B.; Zhang, Z.-Y.; Kjeldsen, S. E. 2023 ESH Guidelines for the Management of Arterial Hypertension The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension: Endorsed by the International Society of Hypertension (ISH) and the European Renal Association (ERA). *J. Hypertens.* **2023**, *41* (12), 1874−2071.

(2) Whelton, P. K.; Flack, J. M.; Jennings, G.; Schutte, A.; Wang, J.; Touyz, R. M. Editors' Commentary on the 2023 ESH Management of Arterial Hypertension Guidelines. *Hypertension* **2023**, *80* (9), 1795−1799.

(3) Hypertension. https://www.who.int/news-room/fact-sheets/detail/hypertension (accessed February 09, 2024).

(4) Picariello, C.; Lazzeri, C.; Attanà, P.; Chiostri, M.; Gensini, G. F.; Valente, S. The Impact of Hypertension on Patients with Acute Coronary Syndromes. *Int. J. Hypertens.* **2011**, *2011*, No. 563657.

(5) Chatzis, D. G.; Magounaki, K.; Pantazopoulos, I.; Beltsios, E. T.; Katsi, V.; Tsioufis, K. P. Current Management of Hypertension in Older Adults. *Drugs Aging* **2023**, *40* (5), 407−416.

(6) Khalil, H.; Zeltser, R. Antihypertensive Medications. In *StatPearls*; StatPearls Publishing: Treasure Island (FL), 2023.

(7) Appel, L. J.; Champagne, C. M.; Harsha, D. W.; Cooper, L. S.; Obarzanek, E.; Elmer, P. J.; Stevens, V. J.; Vollmer, W. M.; Lin, P.-H.; Svetkey, L. P.; Stedman, S. W.; Young, D. R.; Writing Group of the PREMIER Collaborative Research Group. Effects of Comprehensive Lifestyle Modification on Blood Pressure Control: Main Results of the PREMIER Clinical Trial. *JAMA* **2003**, *289* (16), 2083−2093.

(8) Aburto, N. J.; Ziolkovska, A.; Hooper, L.; Elliott, P.; Cappuccio, F.; Meerpohl, J. Effect of Lower Sodium Intake on Health: Systematic Review and Meta-Analyses. *BMJ* **2013**, *346*, No. f1326.

(9) Cornelissen, V. A.; Smart, N. A. Exercise Training for Blood Pressure: A Systematic Review and Meta-Analysis. *J. Am. Heart Assoc.* **2013**, *2* (1), No. e004473.

(10) Neter, J. E.; Stam, B. E.; Kok, F. J.; Grobbee, D. E.; Geleijnse, J. M. Influence of Weight Reduction on Blood Pressure: A Meta-Analysis of Randomized Controlled Trials. *Hypertension* **2003**, *42* (5), 878−884.

(11) Verma, N.; Rastogi, S.; Chia, Y.-C.; Siddique, S.; Turana, Y.; Cheng, H.; Sogunuru, G. P.; Tay, J. C.; Teo, B. W.; Wang, T.-D.; Tsoi, K. K. F.; Kario, K. Non-pharmacological Management of Hypertension. *J. Clin. Hypertens.* **2021**, *23* (7), 1275−1283.

(12) Grøn sælen, M.; Prøsch, L. K.; Gudmundsdottir, H.; Dyrbekk, D.; Helge Hunderi, O.; Arnesen, E.; Paulsen, D.; Skjønsberg, H.; Os, I. Controlling Systolic Blood Pressure Is Difficult in Patients with Diabetic Kidney Disease Exhibiting Moderate-to-Severe Reductions in Renal Function. *Blood Pressure* **2005**, *14* (3), 170−176.

(13) Carey, R. M.; Wright, J. T.; Taler, S. J.; Whelton, P. K. Guideline-Driven Management of Hypertension: An Evidence-Based Update. *Circ. Res.* **2021**, *128* (7), 827−846.

(14) Calhoun, D. A.; Jones, D.; Textor, S.; Goff, D. C.; Murphy, T. P.; Toto, R. D.; White, A.; Cushman, W. C.; White, W.; Sica, D.; Ferdinand, K.; Giles, T. D.; Falkner, B.; Carey, R. M. Resistant Hypertension: Diagnosis, Evaluation, and Treatment. *Circulation* **2008**, *117* (25), e510−e526.

(15) Avataneo, V.; De Nicolò, A.; Rabbia, F.; Perlo, E.; Burrello, J.; Berra, E.; Pappaccogli, M.; Cusato, J.; D'Avolio, A.; Di Perri, G.; Veglio, F. Therapeutic Drug Monitoring-Guided Definition of Adherence Profiles in Resistant Hypertension and Identification of Predictors of Poor Adherence. *Br. J. Clin. Pharmacol.* **2018**, *84* (11), 2535−2543.

(16) Rabbia, F.; Fulcheri, C.; Di Monaco, S.; Covella, M.; Perlo, E.; Pappaccogli, M.; Veglio, F. Adherence to Antihypertensive Therapy and Therapeutic Dosage of Antihypertensive Drugs. *High Blood Pressure Cardiovasc. Prev.* **2016**, *23* (4), 341−345.

(17) Claros, N. M. Importance of Adherence in the Management of Hypertension. *Hipertens. Riesgo Vasc.* **2023**, *40* (1), 34−39.

(18) Kang, J.-S.; Lee, M.-H. Overview of Therapeutic Drug Monitoring. *Korean J. Int. Med.* **2009**, *24* (1), 1−10.

(19) Fang, Z.; Zhang, H.; Guo, J.; Guo, J. Overview of Therapeutic Drug Monitoring and Clinical Practice. *Talanta* **2024**, *266*, No. 124996.

(20) Wagmann, L.; Vollmer, A. C.; Lauder, L.; Mahfoud, F.; Meyer, M. R. Assessing Adherence to Antihypertensive Medication by Means of Dose-Dependent Reference Plasma Concentration Ranges and Ultra-High Performance Liquid Chromatography-Ion Trap Mass Spectrometry Analysis. *Molecules* **2021**, *26* (5), No. 1495.

(21) De Nicolò, A.; Avataneo, V.; Rabbia, F.; Bonifacio, G.; Cusato, J.; Tomasello, C.; Perlo, E.; Mulatero, P.; Veglio, F.; Di Perri, G.; D'Avolio, A. UHPLC−MS/MS Method with Protein Precipitation Extraction for the Simultaneous Quantification of Ten Antihypertensive Drugs in Human Plasma from Resistant Hypertensive Patients. *J. Pharm. Biomed. Anal.* **2016**, *129*, 535−541.

(22) Avataneo, V.; Fanelli, E.; De Nicolò, A.; Rabbia, F.; Palermiti, A.; Pappaccogli, M.; Cusato, J.; De Rosa, F. G.; D'Avolio, A.; Veglio, F. A Non-Invasive Method for Detection of Antihypertensive Drugs in Biological Fluids: The Salivary Therapeutic Drug Monitoring. *Front. Pharmacol.* **2022**, *12*, No. 755184.

(23) Thorstensen, C. W.; Clasen, P.-E.; Rognstad, S.; Haldsrud, R.; Føreid, S.; Helstrøm, T.; Bergland, O. U.; Halvorsen, L. V.; Aune, A.; Olsen, E.; Brobak, K. M.; Høieggen, A.; Gustavsen, I.; Larstorp, A. C. K.; Søraas, C. L.; Opdal, M. S. Development of UHPLC-MS/MS Methods to Quantify 25 Antihypertensive Drugs in Serum in a Cohort of Patients Treated for Hypertension. *J. Pharm. Biomed. Anal.* **2022**, *219*, No. 114908.

(24) De Nicolò, A.; Avataneo, V.; Rabbia, F.; Sciandra, M.; Tosello, F.; Cusato, J.; Perlo, E.; Mulatero, P.; Veglio, F.; Di Perri, G.; D'Avolio, A. UHPLC-MS/MS Method with Sample Dilution to Test Therapeutic Adherence through Quantification of Ten Antihypertensive Drugs in Urine Samples. *J. Pharm. Biomed. Anal.* **2017**, *142*, 279−285.

(25) *Guideline for the Pharmacological Treatment of Hypertension in Adults*, WHO Guidelines Approved by the Guidelines Review Committee; World Health Organization: Geneva, 2021.

(26) Ich-Guideline-M10-Bioanalytical-Method-Validation-Step-5_en.Pdf. https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-m10-bioanalytical-method-validation-step-5_en.pdf (accessed September 17, 2024).

(27) Bioanalytical Method Validation Guidance for Industry. 2018.

https://doi.org/10.1021/acs.jmedchem.4c02045
J. Med. Chem. 2024, 67, 22124−22133

(28) M10 Bioanalytical Method Validation and Study Sample Analysis.
(29) Bönner, G.; Landers, B.; Bramlage, P. Candesartan Cilexetil/Hydrochlorothiazide Combination Treatment versus High-Dose Candesartan Cilexetil Monotherapy in Patients with Mild to Moderate Cardiovascular Risk (CHILI Triple T). *Vasc. Health Risk Manag.* **2011**, *7*, 85−95.
(30) Luna, R. L.; Oigman, W.; Ramirez, J. A.; Mion, D.; Batlouni, M.; da Rocha, J. C.; Feitosa, G. S.; Castro, I.; de C Chaves Júnior, H.; God, E. M.; Maia, L. N.; Ortega, K. C.; Raineri, A. M. Efficacy and tolerance of the bisoprolol/hydrochlorothiazide combination in arterial hypertension. *Arq. Bras. Cardiol.* **1998**, *71* (4), 601−608.
(31) Fujikawa, K.; Hasebe, N.; Kikuchi, K.; NICE-Combi Study Group. Cost-Effectiveness Analysis of Hypertension Treatment: Controlled Release Nifedipine and Candesartan Low-Dose Combination Therapy in Patients with Essential Hypertension–the Nifedipine and Candesartan Combination (NICE-Combi) Study. *Hypertens. Res.* **2005**, *28* (7), 585−591.
(32) Forjan, V.; Maričič, L. C.; Prosen, H.; Vončina, D. B. Determination of Candesartan in Human Plasma with Liquid Chromatography - Tandem Mass Spectrometry. *Acta Chim. Slov.* **2016**, *63* (1), 38−46.
(33) Abdel-Megied, A. M.; Kovalenko, S.; Elbarbry, F. A.; Piponski, M.; Oleshchuk, O.; El Deeb, S.; Magdy, G.; Belal, F.; Grochovuy, T.; Logoyda, L. LC-MS/MS Bioanalytical Method for the Quantitative Analysis of Nifedipine, Bisoprolol, and Captopril in Human Plasma: Application to Pharmacokinetic Studies. *Biomed. Chromatogr.* **2023**, *37* (9), No. e5664.
(34) Ding, L.; Zhou, X.; Guo, X.; Song, Q.; He, J.; Xu, G. LC−ESI-MS Method for the Determination of Bisoprolol in Human Plasma. *J. Pharm. Biomed. Anal.* **2007**, *44* (2), 520−525.
(35) Zisaki, A.; Miskovic, L.; Hatzimanikatis, V. Antihypertensive Drugs Metabolism: An Update to Pharmacokinetic Profiles and Computational Approaches. *Curr. Pharm. Des.* **2014**, *21* (6), 806−822.
(36) Arnett, D. K.; Claas, S. A. Pharmacogenetics of Antihypertensive Treatment: Detailing Disciplinary Dissonance. *Pharmacogenomics* **2009**, *10* (8), 1295−1307.
(37) Patriarca, G.; Schiavino, D.; Nucera, E.; Colamonico, P.; Montesarchio, G.; Saraceni, C. Multiple Drug Intolerance: Allergological and Psychological Findings. *J. Invest. Allergol. Clin. Immunol.* **1991**, *1* (2), 138−144.
(38) Polaczyk, M.; Olszanecka, A.; Wojciechowska, W.; Rajzer, M.; Stolarz-Skrzypek, K. Multiple Drug Intolerance in Patients with Arterial Hypertension: Prevalence and Determining Factors. *Pol. Arch. Intern. Med.* **2023**, *133* (3), No. 16399.
(39) Q2 (R1) Validation of Analytical Procedures: Text and Methodology 2006.
(40) Taylor, P. J. Matrix Effects: The Achilles Heel of Quantitative High-Performance Liquid Chromatography-Electrospray-Tandem Mass Spectrometry. *Clin. Biochem.* **2005**, *38* (4), 328−334.
(41) De Nicolò, A.; Cantu, M.; D'Avolio, A. Matrix Effect Management in Liquid Chromatography Mass Spectrometry: The Internal Standard Normalized Matrix Effect. *Bioanalysis* **2017**, *9*, 1093−1105.

https://doi.org/10.1021/acs.jmedchem.4c02045
J. Med. Chem. 2024, 67, 22124−22133