# Development of UHPLC-MS/MS methods to quantify 25 antihypertensive drugs in serum in a cohort of patients treated for hypertension

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## ARTICLE INFO

## ABSTRACT

We developed three ultra-high pressure liquid chromatography coupled to mass spectrometry detection (UHPLC-MS/MS) methods to quantify 25 antihypertensive drugs in serum samples. Patient-reported drug lists were collected, and drug concentrations were analysed in samples from 547 patients, half with uncontrolled hypertension, and all treated with $\geq 2$ antihypertensive drugs.

For sample preparation, serum was mixed with deuterated internal standards and acetonitrile and precipitated. Aliquots of the supernatant were injected on UHPLC-MSMS with a C18 reversed phase column. The mobile phase was 0.1 % HCOOH (formic acid) in water and 0.1 % HCOOH in acetonitrile (except in methanol for spironolactone/canrenone) at a flow rate of 0.4 mL/min. The calibrators and internal controls were prepared in Autonorm™. The calibration ranges were wide, and the models were linear or quadratic with squared correlation coefficients $\geq 0.97$. The limits of detection and quantification, specificity, carry-over, and matrix effects were acceptable. The accuracy of the internal controls was in the range 85–121 %, and the intermediate precision for all drugs was 4–28 %.

The patient-reported antihypertensive drug use and the detected serum drug concentrations were in accordance with that most frequently prescribed nationally. The percent non-detectable level was 5–10 % for bendroflumethiazide, doxazosin, nifedipine, and ramipril. Often the drug dose chosen was lower than the recommended maximum daily dose. We report the maximum ($C_{max}$) and minimum ($C_{min}$) drug concentrations after drug intake. The inter-individual pharmacokinetic variability at $C_{min}$ was 18-fold for hydrochlorothiazide, 22-fold for losartan carboxyl acid, 26-fold for amlodipine, 44-fold for candesartan, and 50-fold for valsartan.

Our methods are suitable for measuring antihypertensive drugs in patient serum for therapy control.

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## 1. Introduction

Selective alpha-blockers, angiotensin-converting enzyme inhibitors (ACEIs), angiotensin II receptor blockers (ARBs), beta-blockers, calcium channel blockers (CCBs), and diuretics (usually thiazides and much more seldom loop diuretics and potassium-saving diuretics), referred to as antihypertensive drugs, are medication prescribed to treat hypertension [1].

Hypertension is a major risk factor for morbidity and mortality worldwide [2], and about 25–30 % of the general adult population in Norway has increased blood pressure. However, up to half of the patients do not achieve a normal blood pressure despite lifestyle changes [3] and prescription of antihypertensive drugs, and this is referred to as uncontrolled hypertension. Uncontrolled hypertension can be caused by poor drug adherence and/or inadequate pharmacological treatment associated with pharmacokinetic and pharmacodynamic variation. The study by Berra et al. reported that 23–66 % of all patients with hypertension are non-adherent/partially adherent to their prescribed drugs [4], while others report a non-adherence level with prevalence of 7–10 % [1,5].

The gold standard for therapy control in hypertension is blood pressure measurements. Drug intake can be controlled by directly observed drug intake, by electronic recording devices [6,7], by questionnaires [8] or more objectively by measuring antihypertensive drugs in serum/plasma and/or urine [9]. Measurements of serum drug concentrations together with drug responses are termed therapeutic drug monitoring. If the serum concentrations have not been directly associated with drug responses, they are instead defined as dose-related reference ranges, and can be based on pharmacokinetic calculations and/or on drug serum measurements from samples collected at standardized time after last drug intake. In a previous study we established dose-related reference ranges mostly based on pharmacokinetic calculations for 24 antihypertensive drugs [10], and we have recently published cut-off values defining drug non-adherence [5].

A systematic review and meta-analysis from 2016 showed that several methods using liquid chromatography coupled to mass spectrometry (LC-MS/MS) detection have been developed to measure antihypertensive drugs in serum, plasma, and urine from hypertensive patients [9]. Both urine and serum/plasma can be applied to assess drug adherence [11,12]; however, concentrations in the urine have a long detection time window relating only to drug intake and not to clinical response, and detailed knowledge of the excretory pattern of the drug is necessary to assess the time since intake. Analyses in serum have a short time window of detection but are more suitable for drug adherence studies than urine because the drug serum/plasma concentration is associated with the blood pressure-reducing effect [13].

LC-MS/MS methods with four different sampling preparation techniques have been reported, but the time durations and steps in the procedures vary. The methods reported by Kristoffersen et al., Iriarte et al., and Florczak et al. use solid phase extraction [14–16], while another technique is liquid-liquid extraction [17,18]. Some authors have described the removal of plasma proteins by precipitation followed by nitrogen steam evaporation with or without increasing the concentration in the sample preparation step [13, 19–21], while others have only performed a precipitation step as the sampling procedure or used an extraction column [22–26]. Our goal was a simple and short sample preparation to reduce the time and effort required and to avoid drug loss during the procedure.

This paper describes how we developed three methods using UHPLC-MS/MS with a simple sample preparation to measure the serum levels of the 25 most commonly prescribed antihypertensive drugs in Norway [27] and differed slightly from the selection of drugs in other developed methods [19–21, 24, 28]. Our methods were developed to analyse serum samples in a clinical hypertension research project. The methods were used to compare patient-reported drug lists and the serum drug concentrations from a cohort of 547 patients treated for hypertension. The results were examined and compared with the prescribed antihypertensive drugs in Norway in 2018. Further, we determined the percentage of patients with undetectable drug levels in serum for each drug and the percentage of patients using the recommended maximum daily dose. Finally, we defined drug concentrations measured in serum samples close to maximum ($C_{max}$) and minimum ($C_{min}$) concentration after last drug intake.

## 2. Materials and methods

### 2.1. Antihypertensive drug selection

We searched the Norwegian Prescription Database from 2014 to 2017 in order to identify the most prescribed antihypertensive drugs. Dose-related reference ranges were established as previously reported [10].

### 2.2. Chemicals

The drugs and deuterium-labelled drugs were mainly purchased from Chiron Pharmasynth AS (Trondheim, Norway), while labetalol, lercandipine, losartan carboxylic acid and ramiprilat for calibrators, and their deuterium labelled drugs were purchased from Alsachim (Illkirch-Graffenstaden, France) and the corresponding controls from Sigma (St. Louis, USA). The drugs from Chiron were already dissolved in 100 % methanol with the concentration of 1 mg/mL. The drugs from Alsachim and Sigma contain 1 mg dry powder which was dissolved in 1 mL of 100 % methanol to the concentration 1 mg/mL. Methanol (LC/MS grade) was purchased from Fisher Scientific (Oslo, Norway) or Merck (Darmstadt, Germany), acetonitrile was purchased from Merck (Darmstadt, Germany), and HPLC-grade water was from a Milli-Q-water purification system (Darmstadt, Germany). Autonorm™ was from Sero AS (Oslo, Norway), and serum vacutainers without substituents were purchased from BD (New Jersey, USA). Microtubes (2 mL, 72694) for freezing samples, standards, and controls were purchased from Sarstedt AG & Co. Kh, (Nümbrecht, Germany).

### 2.3. Preparation of internal standard solutions

Most of the compounds had their own deuterated internal standard (IS), while deuterated spironolactone was used for canrenone, deuterated ramiprilat was used for labetalol, and deuterated bendroflumethiazide was used for eplerenone. IS working solutions were prepared for each method in methanol as mixtures of all IS compounds in the range of 2.5–5000 nmol/L by diluting IS stock solutions to final concentration.

### 2.4. Sample preparation

A total of 50 μL of serum was mixed for 5–10 s on a vortex mixer with 25 μL IS solution and 150 μL acetonitrile (LCMS-grade). The mixture was then centrifuged for 10 min at 3500 rpm. Aliquots of the supernatant were transferred to vials with inserts, capped, and placed in the autosampler for analysis.

### 2.5. Preparation of calibrators and controls

Two different manufacturers or lots were purchased for the stock solutions of the calibrators and the controls to reveal possible errors in the stock solution preparation and dilution. In addition, two different persons were involved in the process of making the calibrators and controls. For each of the three methods, stock solutions of calibrators and controls prepared in methanol (100 %) were diluted in methanol: $H_2O$ (50:50 v/v) in combinations into three sets of working solutions for calibrators and three for controls. Further dilution to six working solutions for the calibrators and six for the controls was performed. The

**Table 1**
Selected drugs, drug half-lives, serum concentration ranges and the three analytical methods.

| Antihypertensive drugs | T1/2 (h)* | Serum concentration range (nmol/L)** | LOD nmol/L | LOQ nmol/L | Calibrators nmol/L | Calibration curve | Weighting | $R^2$* | ME (%) | Methods |
|---|---|---|---|---|---|---|---|---|---|---|
| Amlodipine | 35–50 | 5–70 | 0.65 | 2.15 | 1–10–50–250–600–1000 | Q | $1/x^2$ | 0.999 | 6.9 | A |
| Atenolol | 6–14 | 75–750 | 3.12 | 10.49 | 20–100–1000–20000–40000–60000 | Q | $1/x$ | 0.998 | 0.6 | B3 |
| Bendroflumethiazide | 3–9 | 2,5–50 | 2.08 | 6.92 | 10–100–200–300–400–500 | Q | $1/x$ | 0.995 | 4.5 | B3 |
| Bisoprolol | 10–13 | 10–200 | 0.06 | 0.20 | 1–10–50–250–600–1000 | Q | $1/x^2$ | 0.999 | 3.7 | A |
| Bumetanide | 1–2 | 100–400*** | 0.87 | 2.89 | 10–100–200–300–400–500 | Q | $1/x$ | 0.999 | 3.7 | B3 |
| Candesartan | 9 | 15–300 | 0.48 | 1.61 | 1–10–50–250–600–1000 | Q | $1/x^2$ | 0.987 | 4.6 | A |
| Canrenone | 9–24 | 30–300 | 0.13 | 0.44 | 1–10–100–400–700–1000 | L | $1/x^2$ | 0.996 | 3.4 | B2 |
| Carvedilol | 6–10 | 5–100 | 0.21 | 0.70 | 1–10–50–250–600–1000 | Q | $1/x^2$ | 0.999 | 2.9 | A |
| Diltiazem | 4–10 | 150–500 | 0.15 | 0.53 | 10–25–50–250–500–1000 | L | $1/x^2$ | 0.999 | 2.9 | C |
| Doxazosin | 9–15 | 10–120 | 0.40 | 1.32 | 10–25–50–100–250–500 | L | $1/x^2$ | 0.998 | 2.0 | C |
| Enalaprilat | 11 | 10–300 | 1.81 | 6.04 | 1–10–50–250–600–1000 | Q | $1/x^2$ | 0.991 | 2.4 | A |
| Eplerenone | 3–6 | 3.5–400 | 0.55 | 3.37 | 20–100–1000–2000–4000–6000 | Q | $1/x^2$ | 0.989 | 7.0 | B3 |
| Furosemide | 1–2 | 6047–30238*** | 65.68 | 218.93 | 1000–2500–5000–10000–25000–50000 | L | $1/x^2$ | 0.999 | 7.5 | A |
| Hydrochlorothiazide | 9–13 | 10–300 | 5.36 | 17.88 | 10–50–250–750–1500–3000 | Q | $1/x^2$ | 0.986 | 4.3 | A |
| Irbesartan | 11–15 | 400–4000 | 4.24 | 14.14 | 240–1200–3000–5000–10000–15000–20000 | Q | $1/x^2$ | 0.970 | 1.4 | B1 |
| Labetalol | 5–8 | 50–1000 | 0.12 | 0.39 | 1–10–50–250–600–1000 | Q | $1/x^2$ | 0.996 | 0.5 | A |
| Lercanidipine | 8–10 | 0.15–5 | 0.04 | 0.12 | 1–5–10–20–50–100 | Q | $1/x^2$ | 0.997 | 4.9 | A |
| Lisinopril | 12 | 10–300 | 6.56 | 21.87 | 10–100–200–300–400–500 | Q | $1/x$ | 0.999 | ˆ | B3 |
| Losartan carboxylic acid | 6–9 | 30–300 | 1.64 | 5.47 | 10–50–250–750–1500–3000 | Q | $1/x^2$ | 0.999 | 0.7 | A |
| Metoprolol | 3–4 | 10–500 | 0.99 | 3.29 | 10–50–250–750–1500–3000 | L | $1/x$ | 0.997 | 5.2 | A |
| Nifedipine | 6–11 | 10–150 | 0.13 | 0.45 | 1–10–50–250–600–1000 | Q | $1/x^2$ | 0.999 | 5.6 | A |
| Ramiprilat | 13–17 | 4–100 | 2.28 | 7.60 | 1–10–50–250–600–1000 | Q | $1/x^2$ | 0.999 | 4.1 | A |
| Telmisartan | 18–30 | 10–200 | 0.04 | 0.12 | 4–20–100–1000–2000–4000–6000 | Q | $1/x$ | 0.999 | 0.6 | B1 |
| Valsartan | 5–9 | 100–4000 | 4.10 | 13.66 | 240–1200–3000–5000–10000–15000–20000 | Q | $1/x^2$ | 0.998 | 2.6 | B1 |
| Verapamil | 4–12 | 50–400 | 0.22 | 0.73 | 9–23–46–231–462–924 | L | $1/x^2$ | 0.999 | 3.9 | C |

ME: Mean bias matrix effects, Q: quadratic, L: linear. * Half-life (T1/2) [30,31]. ** C12–24 serum concentration ranges. *** Serum reference ranges for furosemide and bumetanide represent Cmax values [30,32]. ˆ No data available. LOD = limit of detection and LOQ = limit of quantification, calibrator concentrations, equations of the calibration curves, weighting, R2, matrix effects are shown for the three methods. For the method B three sample injections were necessary, indicated as B1-B3. Matrix effect was determined at calibrator 2 level for each drug.

working solutions for standards and controls were tested before the final preparation of calibrators and controls to ensure they contained the same concentration of analytes. The final calibrators and controls were made by adding working solution to Autonorm™ in order to reach final concentrations. Autonorm™ from Sero was chosen as matrix because it resembles human serum and it is a certified material in which the known amounts of constituents are constant compared to human serum. For each drug six calibrator levels and three control levels (low, medium, and high) were made. Aliquots of each calibrator/control were stored at –80 ºC in darkness to avoid photodegradation of nifedipine, thawed before use and further treated as described in the sample preparation section.

### 2.6. UHPLC-MS/MS

The samples were analysed on an Agilent 1290 Infinity UHPLC combined with an Agilent 6490 MS/MS (Matriks, Oslo, Norway). The analytes were separated on a BEH C-18 reversed phase column (100 mm × 2.1 mm with a 1.7 μm particle size). 2 μL of each serum sample was injected. The drugs were divided in two different methods, A (13 mainly most prescribed drugs) and B (9 less prescribed drugs). The decision to add the three more seldom prescribed drugs diltiazem, doxazosin and verapamil was late in method development and therefore introduced method C, see Table 1. In method A, for eight drugs in method B and method C the mobile phase A1 was 0.1 % HCOOH in water (1 mL HCOOH to 1000 mL water, pH = 3.2) and B1 was 0.1 % HCOOH in acetonitrile (1 mL HCOOH to 1000 mL acetonitrile (100 %)), with a flow rate of 0.4 mL/min. Because spironolactone and canrenone have the same multiple reaction monitoring (MRM) transition, we changed to the more polar solvent B1 of 0.1 % HCOOH in methanol (1 mL HCOOH to 1000 mL methanol (100 %)) to be able to adequately separate spironolactone from canrenone. Irbesartan, telmisartan and valsartan have a much higher upper limit of quantification than the other drugs in method B. Due to this we reduced the electron multiplier voltage (EMV) and the injection volume to 1 μL, carefully to avoid saturation at the high calibrator concentrations. To analyse all drugs in method B the same sample had to be injected three times. Hence, to measure all 25 drugs in our methods we ended up with five injections of the sample. For gradients, mobile phases and time of analysis, see Table A1.

Mass spectrometry detection was performed using an Agilent 6490 triple quadrupole (QQQ) (Matriks, Oslo, Norway) with an electrospray ionization (ESI) source operating in either positive or negative mode using selected multiple reaction monitoring (MRM) windows, see Table A2. The ESI spray set-up was: capillary tension 2000 V, gas flow 14 L/min at 240 °C, sheath gas flow 11 L/min at 350 °C, and nebulizer operated at 20 psi. Precursor ions were transferred inside the first quadrupole with the ion funnel radio frequency high pressure set to 150 V, and 90 V for positive and negative ions, respectively and low pressure to 60 V for both positive and negative ions. Fragmentor voltage was 380 V for all analytes. Collision-induced dissociation (CID) of each precursor ion was performed with $N_2$ as collision gas. The collision energy (eV) was adjusted to optimize the signal for the most abundant product ions, which were subsequently used for MRM analysis. Dynamic MRM was performed to optimize MRM-window and dwell time for each analyte.

### 2.7. Method validation

The methods were validated according to the guidelines of the European Medicine Agency (EMA) [29]. The y-axis of the calibration curve was the ratio of the peak area of the actual drug to its internal standard. Controls (low, medium, and high) for each drug were run in six parallels measurements for three consecutive days. We report the fitting (quadratic or linear) of the calibration curve, the weighting and squared correlation coefficients of the model along with the accuracy (in percent of the spiked value), *bias % = accuracy % − 100 %,* intermediate precision (as percent coefficient of variation (*CV= SD/mean * 100*) and

$analytical\ uncertainty = 2 * \sqrt{bias^2 + CV^2}$ ) of the controls. The limit of detection (LOD) was defined as

$$LOD = \frac{(3 \quad * \quad concentration \quad of \quad calibrator \quad 1)}{\frac{Signal}{noise} \quad at \quad calibrator \quad 1}, \tag{1}$$

and the limit of quantification (LOQ) was defined as

$$LOQ = \frac{(10 \quad * \quad concentration \quad of \quad calibrator \quad 1 \quad )}{\frac{signal}{noise} \quad at \quad calibrator \quad 1}. \tag{2}$$

The mean of 5–13 parallel measurements for each drug is reported.

To test for specificity, the highest concentrations of calibrators for other drugs measured in serum in our laboratory – including opioids, central nervous stimulants, benzodiazepines, antidepressants, antiepileptic drugs, and metformin – were analysed and reported in bias % of calibrator 1 of each drug. Carry-over was tested by measuring parallels of blank samples before (for subtraction from the blank after the calibrator) and after the highest calibrator concentrations for each antihypertensive drug and reported in bias % of calibrator 1 for each drug. To test for matrix effects two replicates of six patient serum samples spiked with the same concentration as calibrator 2 were compared with that dissolved in acetonitrile (100 % solution). Matrix effects (ME %), were calculated according to the formula:

$$ME\%, \quad IS-corrected = \left[ \left( \frac{\frac{Response \quad of \quad calibrator \quad 2 \quad in \quad matrix}{Response \quad of \quad IS \quad in \quad matrix}}{\frac{Response \quad of \quad calibartor \quad 2 \quad in \quad acetonitrile}{Response \quad of \quad IS \quad in \quad acetonitrile}} \right) * 100 \right] \tag{3}$$

Results were reported as mean bias ME (%) and % CV from six patients samples.

Stability for five of the compounds in our methods, bumetanide, eplerenone, furosemide, labetalol and telmisartan had not been previously tested. Triplets of calibrator 6 (in autonorm) containing the actual substances were analysed after being kept 0, 1, 3 and 10 days at room temperature and in the dark, and thereafter kept at.

-80ºC until all samples were analysed in batch. The results were reported as bias % from calibrator 6 at day 0.

### 2.8. Serum samples

The patient serum samples were from a national hypertension study performed at four university hospitals in Norway and approved by The Regional Committees for Medical and Health Research Ethics and the Data Protection Officer at the Oslo University Hospital in accordance with the Helsinki Declaration. Patients were included if they were ≥ 18 years old, being prescribed ≥ 2 antihypertensive drugs, and were on a stable drug treatment for at least 4 weeks. After signing the written informed consent, remaining uninformed about the intention to screen for non-adherence and collection of serum samples for drug analyses, patients underwent physician-patient interview and examinations. Serum samples were collected from 547 patients. The mean (SD) age of the patients was 63.2 (10.8) years, 42.6 % were women, the body mass index was 29.5 (5.1) kg/m$^2$ and 94.7 % of the patients were Caucasian.

51 % of the patients had uncontrolled hypertension as defined by 24-hrs ambulatory blood pressure monitoring [5]. Complete patient-reported drug lists – including medication, dose, date of treatment initiation, and time since last drug intake – for all antihypertensive drugs were obtained and followed the laboratory requisition. Information of type of concomitant medications was also available. Serum samples were collected at various time intervals after drug intake (range 0.25–76 hrs), but most samples were collected close to $C_{max}$. Serum was sampled from the cubital fossa into vacutainers without substituents.
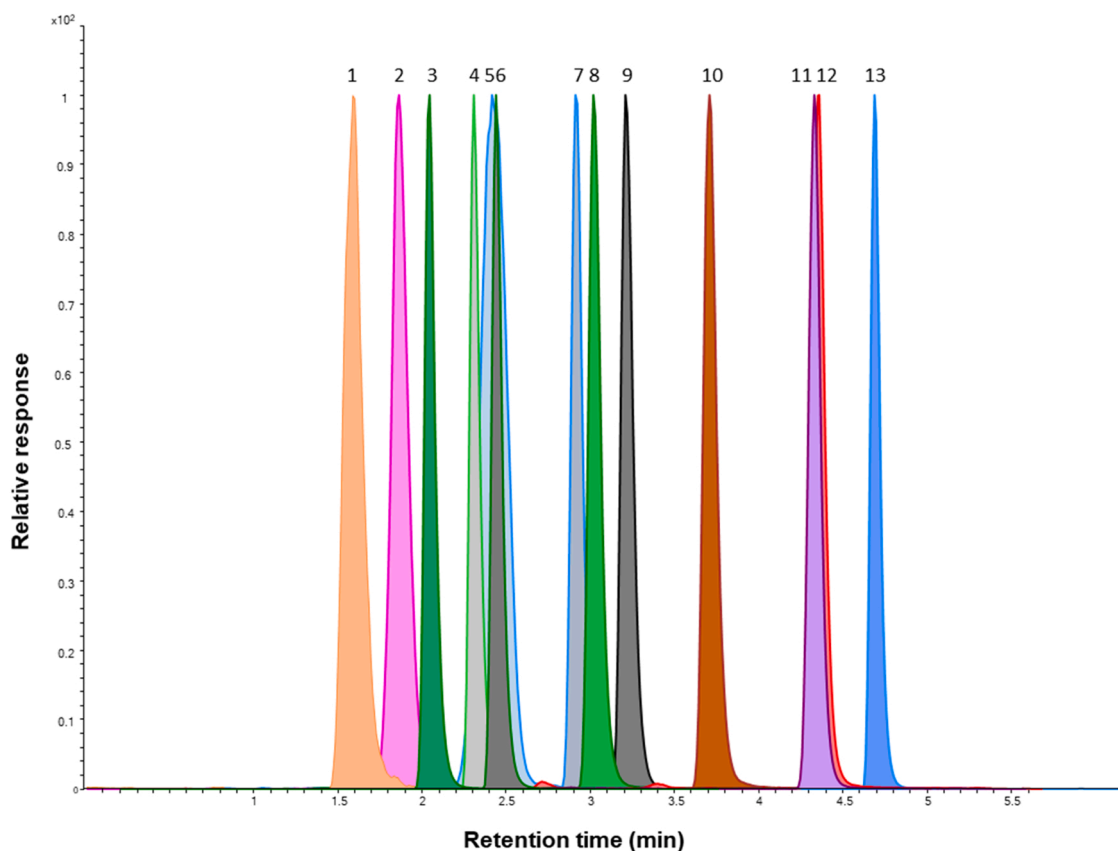
**Fig. 1.** shows a typical chromatogram of method A of 13 of some of the most prescribed antihypertensive drugs: 1) Hydrochlorothiazide, 2) Enaprilat, 3) Metoprolol, 4) Labetalol, 5) Bisoprolol, 6) Ramiprilat, 7) Carvedilol, 8) Furosemide, 9) Amlodipine, 10) Candesartan, 11) Nifedipine, 12) Losartan carboxylic acid, 13) Lercanidipine.

The samples were centrifuged at 3000 rpm and stored at −20 °C in Sarstedt tubes. The samples were kept frozen and protected from light during express transport to the Section of Clinical Pharmacology, Oslo University Hospital. The samples were thawed, prepared as described in *Section 2.4* and analysed in batches by UHPLC-MSMS. The serum drug concentrations were retrieved from the Electronic Data Capture System, VieDoc.

### 2.9. Statistics

Agilent Mass Hunter Qualitative/Quantitative 6.0 software, Microsoft Excel 2010, and IBM SPSS version 26 were used to analyse the data. Regression analysis was used to find the best model fit by linear or quadratic equations. Intermediate precision was calculated from variance analysis. Serum drug measurements from patient samples were reported as median (range) concentrations. We calculated pharmacokinetic variability from the steady state formula:

$$C_{ss} \Big/ \text{daily dose (nmol} \Big/ \text{L} \Big/ \text{mg)} \quad = \frac{F}{di * Cl_{total}} \quad (4)$$

We defined $C_{ss}$ = here as the $C_{12–24}$ concentration at steady state, F = bioavailability, di = the dose interval, and $Cl_{total}$ = total drug clearance.

### 3. Results and discussion

#### 3.1. Selection of antihypertensive drugs

The 25 antihypertensive drugs in our methods covered 97 % of antihypertensive drugs prescribed in Norway to treat hypertension in

2018, as previously reported by Rognstad et al. [10]. Our analytical methods included 1 alpha-blocker, 5 angiotensin II receptor blockers, 3 angiotensin-converting enzyme inhibitors, 5 beta-blockers, 5 calcium channel blockers, and 6 diuretics. For enalapril, losartan, ramipril, and spironolactone, the biologically active metabolites enalaprilat, losartan carboxylic acid, ramiprilat, and canrenone were measured. Table 1, column 1–3 show the selected drugs, their half-lives, and their established dose-related reference ranges at $C_{12–24}$ after drug intake. The established dose-related reference ranges in Table 1 deviated slightly from previously published ranges because fewer patient samples were available for correction of calculated values at the onset of this method development [10].

#### 3.2. Sample preparation

##### 3.2.1. Sample preparation and IS solutions

The sample preparation step in our method only involved a precipitation step like in some previously reported studies [22–26]. Few other methods have reported such short and simple sample preparation for 25 cardiovascular drugs. Deuterated IS solutions for each drug were added to adjust for variation in sample preparation and analysis according to EMA guidelines [29].

##### 3.2.2. Calibrators

The drug calibration ranges were based on the established dose-related reference ranges in Table 1 and should cover both low ($C_{min}$) and high ($C_{max}$) values. The calibrators and internal controls were made in Autonorm™, which resembles serum. For defined LOD, LOQ, calibration ranges, model of fit, weighting, and $R^2$, see Table 1.

### 3.3. Measurement by UHPLC-MS/MS

#### 3.3.1. Chromatogram
Fig. 1 shows a typical chromatogram from method A.

#### 3.3.2. Equation of calibration curve
A linear or quadratic equation model was used to fit the calibrators of each drug and the corresponding squared correlation coefficient of the model as shown in Table 1. The calibrator concentration ranges were wide for many drugs, and therefore a quadratic curve most frequently was applied. The squared correlation coefficient was 0.97 or higher.

#### 3.3.3. Method validation
Six parallels of low, medium, and high control concentrations for each drug were analysed on three different days. The accuracy was in the range 85–121 % and for all drugs at three control levels the intermediate precision range was 4–28 %, see Table A3. Further, our methods showed a mean total analytical uncertainty of about 22 % at all three control levels for all drugs, which was acceptable according to our and others' validation criteria [29]. No commercial external quality programs have been available, but the measured internal control concentration ranges at the three levels of all drugs were within set accepted ranges over the three years of method utilization.

#### 3.3.4. Specificity
The highest calibrator concentrations of other drugs measured in our laboratory gave no responses for most antihypertensive drugs. There was a small response in the range 0.2–4.3 % for nine antihypertensive drugs. As the percentages were very low and calculated from low calibrator level 1 of the drugs, these values could be disregarded. Thus, all results were acceptable according to the validation requirements.

#### 3.3.5. Carry-over
The carry-over was less than 14 % of calibrator 1 for all antihypertensive drugs, except for two drugs. The carry-over was 19 % for telmisartan and 26 % for carvedilol, and we accepted this even though the carry–over requirement should be less than 20 % for all drugs included. When telmisartan and carvedilol were requested for analysis, we added three blank samples after the highest calibrators to wash away the carry-over.

#### 3.3.6. Matrix effect
The bias matrix effect with IS correction was in the range 0.5–7.5 % (Table 1), and CV was in the range 3–11 % for all drugs except for three. The CV was 19 % for bendroflumethiazide, 22 % for ramiprilat, and 24 % for hydrochlorothiazide, all higher than the requirement of 15 %. The results showed that the IS compensated for variation in the matrix. In addition, the calibrators and quality controls were made in Autonorm™, which is serum-like and will also compensate for variation in the matrix.

#### 3.3.7. Stability of drugs in solution and samples
We did not include stability documentation in this study due to published reports showing the stability of solutions and samples at different temperatures [21]. Our methods contain five more compounds than this stability study. Percent bias from time zero of calibrator 6 after 1, 3 and 10 days at room temperature was respectively, 1.6–4.9–19.9 % for bumetanide, (−3.4)- (−1.0)-(−15.2) % for eplerenone, (−5.0)−2.7–5.1 % for furosemide, (−0.4)− 5.0–13.1 % for labetalol, and (−7.1)-(−4.5)− 14.4 % for telmisartan. This shows that the drugs are stable at room temperature for at least three days. Corresponding with the stability results earlier reported [21] we did find that for instance bendroflumethiazide, diltiazem and irbesartan had reduced stability after 3–10 days at room temperature. Because of this, we recommended that the patients samples were sent express to our laboratory frozen and light protrected (calcium blockers are known to light sensitive). At the laboratory we stored all the solutions in the dark to avoid photo degradation. Calibrators and controls were kept frozen at −80 °C until use and patient serum samples were stored at −20 °C until analysis.

### 3.4. Strengths and limitations of the analytical methods

Our results show that simple sample preparation can be performed in order to quantify a large number of different antihypertensive drugs in serum samples ≥ LOQ, see Table 1. Our methods cover wide concentration ranges making them suitable for measuring drug concentrations at both high and low levels. When available in the future, we will join external quality programs. The use of 3 methods and five injections can be a limitation of our method. Since 2020, we have solved this by rather minor adjustments. The analysis is now more robust with 2 injections and 2 methods (A and B) simply by transferring more drugs into method A, transferring drugs in method C to B and further by changing the B1 mobile phase in method B to 0.1 % HCOOH in methanol (1 mL HCOOH to 1000 mL methanol (100 %)) and more dilution of the samples.

Only bendroflumethiazide, hydrochlorothiazide, lisinopril, and ramipril have low established dose-related reference ranges below the LOQ values but still above the LOD. Accordingly, our methods must be applied with caution when assessing the results of patient samples at such low levels for these drugs.

Further, the calibration range of lercanidipine was at too high

**Table 2**
Drug use based on information from patient-reported drug lists and serum drug concentrations.

| Antihypertensive drugs | Number of measurements (% of n = 547) | Number (%) of samples in which the drug was not detected | Highest daily drug dose (mg) in cohort | Number (%) of samples with highest daily dose |
|---|---|---|---|---|
| Hydrochlorothiazide | 327 (60) | 8 (2) | 50 | 3 (1) |
| Amlodipine | 208 (38) | 4 (2) | 10 | 111 (53) |
| Metoprolol | 177 (32) | 3 (2) | 200 | 25 (14) |
| Candesartan | 157 (29) | 3 (2) | 32 | 61 (39) |
| Valsartan | 152 (28) | 3 (2) | 320 | 49 (32) |
| Lercanidipine | 88 (16) | 1 (1) | 20 | 43 (49) |
| Losartan carboxylic acid | 82 (15) | 0 | 100 | 54 (66) |
| Nifedipine | 45 (8) | 3 (7) | 60 | 28 (62) |
| Irbesartan | 43 (8) | 0 | 300 | 34 (79) |
| Enalaprilat | 42 (8) | 0 | 20* | 31 (74) |
| Ramiprilat | 28 (5) | 2 (7) | 10 | 15 (54) |
| Canrenone | 27 (5) | 1 (4) | 100 | 1 (4) |
| Bisoprolol | 26 (5) | 1 (4) | 10* | 5 (20) |
| Furosemide | 23 (4) | ** | 80 | 3 (13) |
| Carvedilol | 19 (4) | 0 | 50 | 4 (21) |
| Doxazosin | 17 (3) | 1 (6) | 8 | 9 (53) |
| Bumetanide | 13 (2) | ** | 4 | 1 (8) |
| Bendroflumethiazide | 10 (2) | 1 (10) | 2.50* | 6 (60) |
| Lisinopril | 9 (2) | 0 | 20* | 8 (89) |
| Telmisartan | 7 (1) | 0 | 80 | 7 (100) |
| Atenolol | 7 (1) | 0 | 100 | 2 (29) |
| Eplerenone | 5 (1) | 0 | 50 | 3 (60) |
| Diltiazem | 5 (1) | 0 | 360* | 1 (20) |
| Verapamil | 3 (1) | 0 | 120* | 3 (100) |
| Labetalol | 1 (0.2) | 0 | 200* | 1 (100) |
| Total number | **1521** | | | |

*The recommended maximum daily dose for enalapril is 40 mg, bisoprolol 20 mg, bendroflumethiazide 5 mg, lisinopril 80 mg, diltiazem 480 mg, verapamil 480 mg, and for labetalol 2400 mg [10].

** not detectable at $C_{min}$

Each patient delivered one serum sample and the analyses were performed based on the patient-reported drug list. Number of requested measurements (%) are listed from most to least frequently used drug in column 2. Number (%) of samples in which no drug was detected are shown in column 3.

In column 4 the highest daily dose corresponded with the recommended maximum daily dose for all drugs except those labelled with *.

**Table 3**

Daily drug doses and serum drug concentrations at defined $C_{max}$ and $C_{min}$ of the 25 drugs.

| Antihypertensive drug | Daily dose in subsets of cohort (mg) | $C_{max}$ | | | $C_{min12-24}$ | | Pharmacokinetic variability |
| | | $T_{max}$ range (h) | n | Serum concentration (nmol/L) | n | Serum concentration (nmol/L) | $C_{min}$ nmol/L/mg dose |
|---|---|---|---|---|---|---|---|
| **Alpha- blocker** | | | | | | | |
| Doxazosin (depot) | 4–8 | 4.5–11.9 | 7 | 60 (24–91) | 3 | 60 (48–85) | |
| **Beta- blockers** | | | | | | | |
| Atenolol | 25–150 | 2–4 | 3 | 1303 (366–2055) | | | |
| Bisoprolol | 1.25–10 | 2–4 | 8 | 52 (18–149) | 5 | 14 (7–34) | |
| Carvedilol | 12.5–50 | 0.5–3 | 3 | 60 (52–176) | | | |
| Labetalol | 200 | 2.7* | 1 | 217 | | | |
| Metoprolol (depot) | 25–200 | 4–11 | 84 | 106 (12–984) | 11 | 59 (13–853) | 1.1 (0.5–5.7) |
| **CCBs** | | | | | | | |
| Amlodipine | 2.5–20 | 2–8 | 169 | 45 (10–172) | 20[a] | 28 (8–105) | 3.7 (0.8–21) |
| Diltiazem | 120–360 | 3–6* | 5 | 412 (246–449) | | | |
| Lercanidipine | 5–30 | 1–4.8 | 46 | 3.8 (1–21.1) | 12[a] | 1.7 (0.4–4.5) | 0.1 (0.04–0.2) |
| Nifedipine (depot) | 30–60 | 2.5–11.9 | 24 | 177 (2–1913) | 6[b] | 100 (33–292) | |
| Verapamil (depot) | 120 | 4–11 | 1 | 205 | 1 | 60 | |
| **ACEIs** | | | | | | | |
| Enalaprilat | 2.5–20 | 2.7–6 | 22 | 254 (43–429) | 2[c] | 13 (9–16) | |
| Lisinopril | 20 | 6–9 | 1 | 207 | 3[a] | 45 (21–45) | |
| Ramiprilat | 1.25–10 | 1–5 | 12 | 68 (4–303) | 3 | 11 (9–15) | |
| **ARBs** | | | | | | | |
| Candesartan | 4–32 | 2–8 | 125 | 454 (39–1733) | 17[b] | 84 (14–490) | 3.2 (0.7–30.6) |
| Irbesartan | 150–300 | 0.5–4 | 17 | 4173 (1922–11017) | 3 | 1250 (592–1923) | |
| Losartan carboxylic acid | 50–100 | 3–5 | 36 | 1041 (124–3109) | 10[a] | 43 (10–216) | 0.7 (0.2–4.3) |
| Telmisartan | 80 | 0.6–3 | 2 | 301 (257–345) | | | |
| Valsartan | 80–320 | 1–4 | 63 | 8013 (50–28605) | 18[a] | 1255 (86–3989) | 7.5 (0.5–25) |
| **Thiazide diuretics** | | | | | | | |
| Bendroflumethiazide | 2.5 | | | | 1 | 10 | |
| Hydrochlorothiazide** | 6–25 | 1–5 | 194 | 285 (42–1604) | 28[b] | 62 (20–352) | 4.2 (1.6–28.2) |
| **Loop diuretics** | | | | | | | |
| Bumetanide | 1 | 1–2 | 1 | 51 | | | |
| Furosemide | 20–80 | 0–5 | 7 | 1878 (298–4011) | | | |
| **Potassium-sparing diuretics** | | | | | | | |
| Canrenone | 12.5–100 | 2–5 | 14 | 109 (23–301) | 3[d] | 52 (13–135) | |
| Eplerenone | 25–50 | 1.5–2 | 2 | 2468 (2455–2480) | 2 | 367 (131–604) | |

* Tmax, reported as the actual available time points because no Tmax values were found in study.

** Values of hydrochlorothiazide below LOQ were excluded.

[a] $C_{12–30}$, [b] $C_{12-32}$, [c] $C_{24-40}$ and [d] $C_{12-27}$

The table shows the daily dose ranges, the applied Tmax levels, the number of samples with Cmax concentrations at defined Tmax and Cmin concentrations for each drug. Serum concentration data are shown as median (range). The pharmacokinetic variability was calculated at $C_{min}$ only for drugs with ≥ 10 samples.

concentration level in method A. Lately, we have changed the calibrator levels of lercanidipine to 0.10–10 nmol/L instead of 1–100 nmol/L. The mean lercanidipine concentration in eight patient samples in the range 0.7–7 nmol/L differed by only 5.9 ± 11.3 % (mean + SD) whether it was analysed by the newly adjusted calibration range or that presented in Table 1.

### 3.5. Measurement of patient serum samples

The presented methods in Table 1 were performed in our laboratory once weekly from October 2017 to October 2020 measuring totally 2203 antihypertensive drugs. Of these, 1521 measurements were from the 547 patient samples in the described hypertension research project. Here we present cross sectional drug use and measurements in the whole population regardless of blood pressure because the sample size was too small for subgrouping.

The 9 most commonly prescribed antihypertensive drugs in Norway in 2018 [27] were also among the 12 most frequently used in the present population, but in a slightly different order, see column 2 of Table 2. Furthermore, Table 2 shows the percentage of patient-reported drug in use but not detected by our methods (below calibrator 1). The highest percentage of patients in which the specific drug was not detected was 10 %, 7.1 %, 6.7 %, and 5.9 % for patients using bendroflumethiazide, ramipril, nifedipine, and doxazosin, respectively, indicating drug non-adherence. For only eight of the 25 drugs (amlodipine, doxazosin,

eplerenone, losartan, irbesartan, nifedipine, ramipril and telmisartan), > 50 % of the patients were prescribed the recommended maximum daily dose. Because the dose levels were submaximal for many drugs prescribed to these patients, it is still an available option for the clinician to increase the drug dose further to achieve a blood pressure reducing effect, especially in the subgroup of patients with uncontrolled hypertension. Though, the current treatment guidelines recommend that most patients are treated with two or more drugs with synergetic effects to reach controlled hypertension and maximal dose levels of each drugs may not be necessary [1].

Table 3 is a subset of the samples shown in Table 2, showing the dose ranges for this subset of samples and steady state serum drug concentrations at defined $C_{max}$ and $C_{min}$ for the 25 drugs. All available $C_{12–24}$ values were in relatively good agreement with the established dose-related reference ranges previously reported by Rognstad et al. and those shown in Table 1 [10]. We report variation in $C_{max}$ values at $T_{max}$, which is the amount of time that a drug is present at the maximum concentration in serum. The $T_{max}$ windows were either ranges or mean ± 1 standard deviations retrieved from Micromedex [30], UpToDate [31], or from additional pharmacokinetic studies (references shown in the appendices).

Drugs administered as depot formulas (doxazosin, metoprolol, nifedipine) and amlodipine and canrenone with rather long half-lives had about the same serum concentrations at $C_{max}$ and $C_{min}$. The ratios of median $C_{max}$/median $C_{min}$ concentrations for these drugs were in the
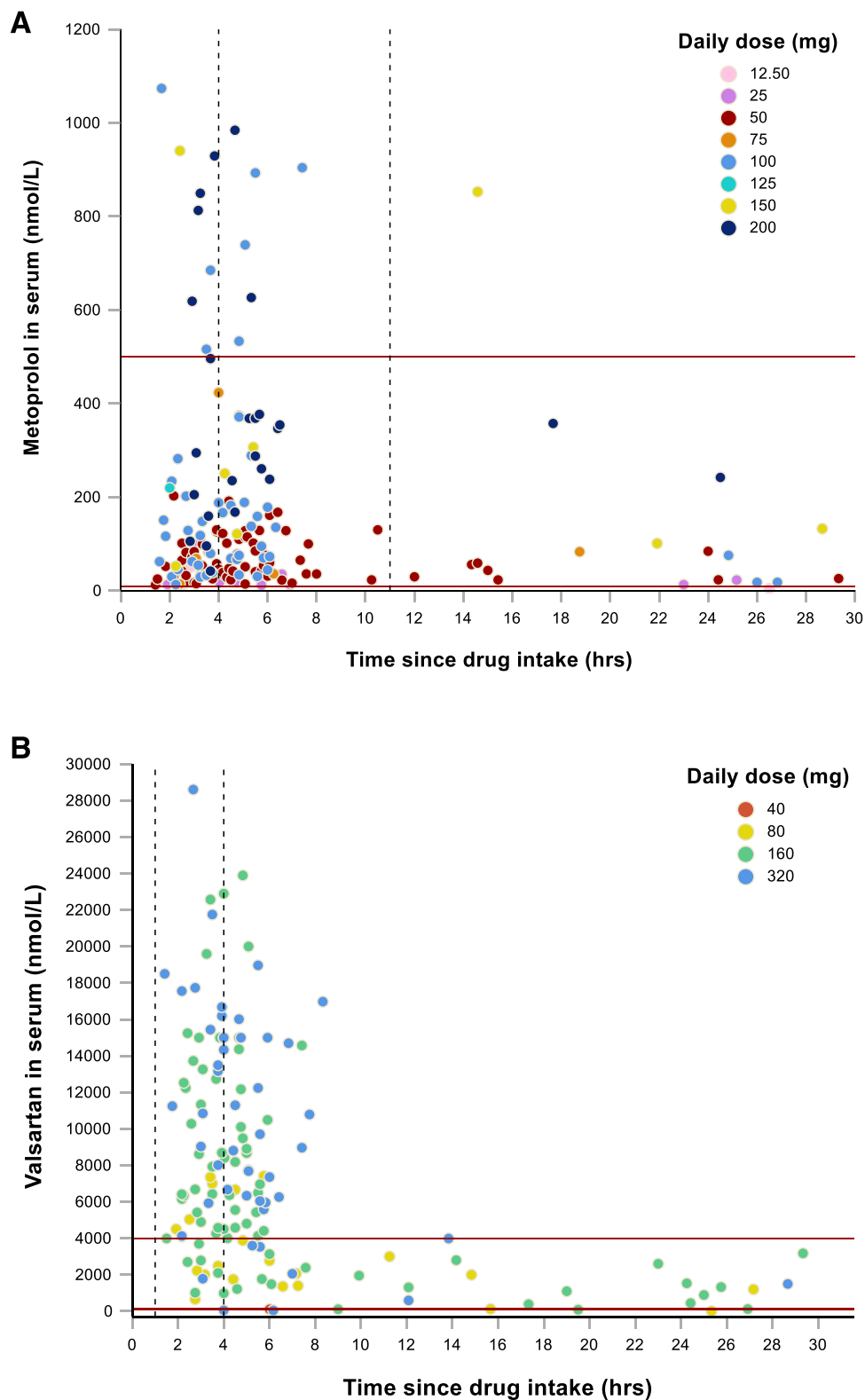
**Fig. 2.** A. Serum drug concentrations of metoprolol, from n = 174 samples, with time after last drug intake. The dotted vertical lines show the $T_{max}$ window of 4–11 h and the horizontal lines are the $C_{12-24}$ serum reference range of 10–500 nmol/L. B. Serum drug concentrations of valsartan, from n = 145 samples, with time after last drug intake. The dotted vertical lines show the $T_{max}$ window of 1–4 hrs and the horizontal lines are the $C_{12-24}$ serum reference range of 100–4000 nmol/L.

range 1–2. Fig. 2A illustrates the distribution for metoprolol with time since last intake and is an example of such a drug. This suggests that serum sampling can be collected anytime in the elimination phase of the dose interval for these drugs. Other drugs had larger ratios between median $C_{max}$/median $C_{min}$, and sampling for therapeutic drug monitoring should therefore always occur 12–24 hrs after drug intake, see Table 3. Fig. 2B illustrates the distribution of valsartan concentration with time since last intake, and is an example of such a drug. Furosemide and bumetanide were measurable only at $C_{max}$ and not at $C_{min}$ by our method as expected due to their short drug half-lives. The serum values

for furosemide at $T_{max}$ 0–5 hrs were lower than that in Table 1; possibly because we allowed values for $C_{max}$ up to 5 hrs, while the only bumetanide value was within the level shown in Table 1 [30,32].

Pharmacokinetic variability was calculated for seven drugs with $\geq 10$ measurements at $C_{min}$, see Table 3. The five highest interindividual pharmacokinetic variability between minimum and maximum values (range), in our study was 18-fold for hydrochlorothiazide, 22-fold for losartan carboxylic acid, 26-fold for amlodipine, 44-fold for candesartan, and 50-fold for valsartan, for ranges see Table 3. However, these findings were based on only 28, 10, 20, 17, and 18 patient samples at $C_{min}$, respectively. For comparison, others report drug concentration variations after single dose intake (however, at $C_{max}$) to be 4-fold for amlodipine, 5-fold for valsartan and 35-fold for losartan carboxylic acid [33]. Measurements can vary because of the shape of the concentration-time curve around $C_{max}$, and therefore it is much safer to define pharmacokinetic variability close to $C_{min}$. None of the drugs in our study showed larger pharmacokinetic variability than 50-fold at $C_{min}$, but the number of samples included in our study is indeed limited and larger studies should be performed to confirm our findings.

Measurement of serum drug concentration has a short time window to detect poor drug adherence, but repeated serum sampling over time can compensate for this. Our methods can be used to measure low concentrations, but also high toxic concentrations to support the diagnosis of intoxication, as reported by Magistrad et al. [34].

Studies are needed to determine whether measurement of serum drug concentrations in patient treated for hypertension can improve clinical outcome in regard to hypertension-mediated organ damage to the brain, heart, and kidneys. Our drug measurements give an overview of a population of 547 patients treated for hypertension, of whom just over half had uncontrolled hypertension. In the future, drug measurements must be compared in patients with controlled and uncontrolled hypertension. Single-pill combinations of two or more drugs are highly recommended for clinical use based on randomized clinical trials with end points of morbidity and mortality [1]. Using single-pill combinations will probably improve pharmacotherapy in hypertension in regard to better drug adherence, but on the other hand it also introduces greater complexity in both dosing and monitoring drug effects, thereby possibly increasing the requirement for measurements of serum drug concentrations as recently suggested by Lane et al. [35].

## 4. Conclusions

Validation of our three developed methods described with simple sample preparation shows that the methods are accurate and precise with no interference or carry-over problems in measuring the selected 25 antihypertensive drugs in serum samples. The methods without concentration steps in the sample preparation must be used with caution at the very low serum drug concentrations for bendroflumethiazide, hydrochlorothiazide, lisinopril, and ramipril. The serum drug concentrations measured in our cohort of 547 hypertensive patients corresponded fairly well with the established $C_{12–24}$ serum concentration ranges. The most frequent drugs not detected in serum were bendroflumethiazide, doxazosin, nifedipine and ramipril (5–10 % of patients). Many patients were not treated with the recommended maximum daily doses. Finally, the pharmacokinetic variability needs to be further studied in larger populations. Our developed methods can be used to measure low and high serum drug concentration and may be a useful tool to help clinicians improve pharmacotherapy in hypertensive patients.

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## Declaration of Competing Interest

The authors confirm no conflict of interest.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2022.114908.

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