High-throughput quantification of 8 antihypertensive drugs and active metabolites in human plasma using UPLC–MS/MS

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ABSTRACT

Background: To assess drug adherence of patients with hypertension, an analytical method was developed and validated using ultra performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS). The method includes eight frequently prescribed antihypertensive drugs from four classes and their active metabolites: angiotensin converting enzyme inhibitors enalapril and perindopril (active metabolites respectively enalaprilate and perindoprilate), angiotensin II receptor blockers losartan (with the active metabolite losartan carboxylic acid) and valsartan, calcium channel blockers amlodipine and nifedipine and diuretics hydrochlorothiazide and spironolactone (with the active metabolite canrenone).

Methods: The antihypertensive drugs were analyzed using a simple and fast sample preparation protocol with protein precipitation followed by chromatographic separation using a gradient elution on a reversed phase column. Mass spectrometric detection was conducted by applying both positive and negative electrospray ionization (ESI+/ESI⁻) and selected reaction monitoring mode (MS/MS). Only 50 μl of plasma sample is needed for the simultaneous quantification of all 12 compounds within 6 min run-to-run analysis time. Enalapril-d5 was applied as internal standard for all compounds except hydrochlorothiazide (internal standard: Hydrochlorothiazide-13C,d2).

Results: The method was validated according to FDA guidelines. Matrix effects were examined using the method of Matuszewski. Correlation coefficients were higher than 0.995 for all compounds. Intra- and inter-day accuracies were < 15% for all analytes except spironolactone (~16.8%) in the established linear range. Intra- and inter-day precision were < 15% for all analytes. As a result of the lower sensitivity of hydrochlorothiazide, the lowest three calibration levels were excluded.

Discussion/Conclusions: The described method is suitable for the simultaneous quantitative analysis of the most commonly used antihypertensive drugs and their corresponding active metabolites. Major advantages are minimal sample volume and clean up and a short runtime. The method is now available to monitor drug adherence of patients with resistant hypertension in our hospital.

1. Introduction

Hypertension is one of the most important risk factors for cardiovascular and renal disease [1]. Despite many drug options for treatment, a considerable number of patients does not reach the target blood pressure. Although there are many reasons for not reaching the target, non-adherence is probably the most important one [2]. Besides blood pressure above target, non-adherence is also independently associated with more hospitalizations and cardiovascular disease [3,4]. It is challenging to define who is non-adherent since differences in drug response make that lack of blood pressure-lowering effect does not automatically mean non-adherence and patients will not always inform their doctors themselves [5]. As a consequence, discussion of non-adherence and available measures to improve adherence are often not applied [6].

Commonly used methods to assess (non-)adherence as self-reporting including usage of validated questionnaires such as the Morisky Medication Adherence Scale or obtaining drug records from the pharmacy lack accuracy [7]. Electronic pill dispensers or observed drug intake are more reliable but costly [8,9]. Therapeutic drug monitoring (TDM) is a reliable and easy to perform way to assess adherence but its use in clinical practice is still limited [10–12]. In a recent pilot study on
TDM in hypertension, informing patients about their undetectable serum drug levels combined with counselling led to improved blood pressure control without a change in treatment regimen [13]. However, quality of the method including determination of the lowest level of quantification needs to be known before it can be used to define (non-)adherence [11,14–16]. Here, we describe a method to measure drug levels for eight commonly used antihypertensive drugs from four classes (angiotensin converting enzyme inhibitors, angiotensin II receptor blockers, calcium channel blockers and diuretics) including four active metabolites. Several methods of analysis for antihypertensive drugs and their corresponding active metabolite in human plasma using LC–MS or LC–MS/MS have been published [17–24]. Most reports describe dedicated methods for only one drug [17,19–24]. Sample volumes ranged from 100 μl to 1 ml. Used sample preparation methods are liquid liquid extraction (LLE) [17,19,21], Solid Phase extraction (SPE) [20,24] and protein precipitation [18,22,23], in some reports combined with evaporation and reconstitution [20,21,23]. Detection methods comprise both selected ion monitoring (SIM) [19] and multiple reaction monitoring (MRM) [17,18,20–24]. Reported ionization modes are positive and negative electrospray ionization [17–21,23,24] or positive atmospheric pressure chemical ionization [19,22].

De Nicolo et al. described a method for the simultaneous quantification of ten antihypertensive drugs [25]. However, we chose a combination of commonly used drugs from four classes including spironolactone which is gaining popularity in resistant hypertension after PATHWAY-2 trial [26]. Therefore, almost every patient with multiple antihypertensive drugs will use at least one drug for which this test is available. To our knowledge, a method of analysis for perindopril, the active metabolite of perindopril, has never been published.

In this assay only 50 μl of sample was used combined with a simple protein precipitation protocol to provide a fast and reliable multi-method for monitoring drug adherence. Chromatographic separation was achieved within 6 min with a gradient elution. Detection was performed by triple quadrupole MS in MRM mode applying positive and negative electrospray ionization.

2. Material and methods

2.1. Chemicals and materials

Amlodipine besylate, hydrochlorothiazide, nifedipine, spironolactone and valsartan were purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). Canrenone, enalapril, enalaprilat-d5 maleate, hydrochlorothiazide-13C2d2, losartan, losartan carboxylic acid, perindopril and perindoprilate were purchased from Santa Cruz Biotechnology Inc (Heidelberg, Germany).

Enalaprilat was purchased from Abcam (Cambridge, United Kingdom).

Methanol absolute LC–MS grade, acetonitrile HPLC Supra-gradient and formic acid 99% ULC/MS grade were purchased from Biosolve BV (Valkenswaard, The Netherlands).

Deionized water was prepared in our laboratory using a Milli-Q Advantage A10 purification system (Merck Millipore, Darmstadt, Germany). Fresh frozen blank human plasma with citrate as anticoagulant was obtained from the hemostasis laboratory of Erasmus MC (Rotterdam, the Netherlands).

2.2. UPLC–MS–MS equipment and conditions

2.2.1. Instrumental

Analysis was carried out on a Thermo TSQ Vantage UPLC–MS/MS system consisting of a Dionex Ultimate UPLC system, connected to a triple Quadrupole mass spectrometer Thermo TSQ Vantage (Thermo Scientific, Waltham, USA). The UPLC system consisted of an Ultimate 3000 RS UPLC-pump, an autosampler and a column oven. Xcalibur™ 2.1(Thermo Scientific), and Chromeleon™ 6.80 (Dionex, Thermo Scientific) were used for data acquisition, controlling respectively the MS/MS and the UPLC-system. LCquan™ 2.6 (Thermo Scientific), was used to process the acquired data.

2.2.2. Chromatographic conditions

Chromatographic separation was achieved using an Acquity UPLC BEH C18 reversed phase column (2.1 × 100 mm, 1.7 μm) (Waters, Milford, USA). Gradient elution was performed with a mobile phase composed of a mixture of 0.1% formic acid in water (eluent A) or methanol (eluent B). A multistep gradient was used at a flow rate of 0.5 ml min$^{-1}$ and was programmed as follows: equilibration at initial conditions with 5% of B, increase to 50% of B from 0 min to 0.1 min, increase to 80% of B from 0.1 min to 2.2 min, increase to 100% of B from 2.2 min to 2.3 min, stabilization at 100% of B from 2.3 min to 2.7 min, back to initial conditions from 2.7 min to 3.0 min equilibration from 3.0 to 5.0 min. The column oven was set at 70 °C. Autosampler temperature was 10 °C.

2.2.3. Mass spectrometry conditions

The MS settings and conditions were as follows: capillary temperature was 250 °C, vaporizer temperature was 400 °C and spray voltage was 3500 V. Sheath and auxiliary nitrogen nebulizer gas pressure were set to 50 and 20 (arbitrary units) respectively. The collision gas pressure (argon) was 1.5 mTorr. The MS run time was 4.0 min performed in the selective reaction monitoring (SRM) scanning mode. Electrospray ionization (ESI) was applied in the positive mode except for hydrochlorothiazide and hydrochlorothiazide-13CD2 (negative mode). The optimized settings of each analyte in Table 1.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Ionization</th>
<th>Precursor ion (m/z)</th>
<th>Product ion (m/z)</th>
<th>Rt (min)</th>
<th>CE (V)</th>
<th>S-lens (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amlodipine</td>
<td>pos.</td>
<td>409.2</td>
<td>238.1</td>
<td>2.35</td>
<td>12</td>
<td>73</td>
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<tr>
<td>Canrenone</td>
<td>pos.</td>
<td>341.3</td>
<td>107.1</td>
<td>2.90</td>
<td>29</td>
<td>114</td>
</tr>
<tr>
<td>Enalapril</td>
<td>pos.</td>
<td>377.3</td>
<td>234.2</td>
<td>2.14</td>
<td>18</td>
<td>102</td>
</tr>
<tr>
<td>Enalapril-d5</td>
<td>pos.</td>
<td>382.3</td>
<td>239.2</td>
<td>2.13</td>
<td>18</td>
<td>102</td>
</tr>
<tr>
<td>Enalaprilate</td>
<td>pos.</td>
<td>349.3</td>
<td>206.2</td>
<td>1.84</td>
<td>17</td>
<td>96</td>
</tr>
<tr>
<td>Hydrochlorothiazide</td>
<td>neg.</td>
<td>296.0</td>
<td>269.0</td>
<td>1.29</td>
<td>−20</td>
<td>−117</td>
</tr>
<tr>
<td>Hydrochlorothiazide-13C2d2</td>
<td>neg.</td>
<td>299.0</td>
<td>270.0</td>
<td>1.28</td>
<td>−22</td>
<td>−117</td>
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<tr>
<td>Losartan</td>
<td>pos.</td>
<td>423.3</td>
<td>207.1</td>
<td>2.60</td>
<td>22</td>
<td>101</td>
</tr>
<tr>
<td>Losartan carboxylic acid</td>
<td>pos.</td>
<td>437.3</td>
<td>207.1</td>
<td>2.76</td>
<td>17</td>
<td>107</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>pos.</td>
<td>347.2</td>
<td>254.2</td>
<td>2.56</td>
<td>17</td>
<td>68</td>
</tr>
<tr>
<td>Perindopril</td>
<td>pos.</td>
<td>369.3</td>
<td>172.2</td>
<td>2.27</td>
<td>20</td>
<td>93</td>
</tr>
<tr>
<td>Perindoprilate</td>
<td>pos.</td>
<td>341.2</td>
<td>170.1</td>
<td>1.93</td>
<td>17</td>
<td>91</td>
</tr>
<tr>
<td>Spironolactone</td>
<td>pos.</td>
<td>341.3</td>
<td>107.1</td>
<td>2.78</td>
<td>29</td>
<td>122</td>
</tr>
<tr>
<td>Valsartan</td>
<td>pos.</td>
<td>436.3</td>
<td>291.2</td>
<td>2.96</td>
<td>16</td>
<td>85</td>
</tr>
</tbody>
</table>
2.3. Standards and quality control samples

2.3.1. Preparation of stock solutions

Two sets of stock solutions for each analyte were prepared at a concentration of 500 mg l\(^{-1}\) in methanol. One set was used for preparation of calibration standards and the other set for preparation of quality control (QC) samples. Stock solutions for the internal standards enalapril-d\(_5\) and hydrochlorothiazide-\(^{13}\)CD\(_2\) were prepared at concentration levels of respectively 100 and 250 mg l\(^{-1}\) in methanol. All stock solutions were stored at \(-18\) °C.

2.3.2. Preparation of calibration standards and quality control samples

A calibration mix solution in 2:1 methanol:water was prepared from the calibration stock solutions of each analyte containing a concentration 20 times higher than the pursued highest concentration of each calibration standard.

The calibration curve was obtained using eight calibration standards. The highest standard was prepared by diluting the calibration mix solution 20 times in blank human plasma. The other seven standards were prepared by diluting the highest standard 2, 4, 10, 40, 100, 400 and 1000 times in blank human plasma. For the purpose of QC samples a quality control mix solution was prepared from the quality control stock solution in the same way as the calibration mix solution. Quality control samples were prepared at three levels; QC L(ow), QC M(edium) and QC H(igh) in blank human plasma by diluting the quality control mix solution respectively 100, 1000 and 4000 times.

For all calibration standards and quality control samples 50 µl aliquots were filled out in 1.5 ml amber colored safe-lock Eppendorf\(^{\circledR}\) tubes and stored at \(-80\) °C prior to analysis.

2.3.3. Preparation of internal standard working solution

The internal standard (IS) working solution was prepared by diluting the stock solution of hydrochlorothiazide-\(^{13}\)CD\(_2\) and enalapril-d\(_5\) respectively 1000 and 4000 times in a mixture of acetonitrile and methanol 1:1 resulting in concentrations of 250 µg l\(^{-1}\) for hydrochlorothiazide-\(^{13}\)CD\(_2\) and 25 µg l\(^{-1}\) for enalapril-d\(_5\). The internal standard solution was stored at \(-20\) °C.

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Fig. 1. Typical SRM Chromatograms of the lowest calibration standard in plasma. Enalapril-d\(_5\) and hydrochlorothiazide-\(^{13}\)CD\(_2\) were used as internal standards.
2.4. Sample preparation

To 50 μl of sample (K2EDTA-plasma) 200 μl of IS working solution was added. After vortexing for 10 s the samples were stored in the refrigerator for 10 min. Subsequently the samples were centrifuged at 16,000 × g for 5 min. The supernatant was transferred to an amber colored autosampler vial and 450 μl of mobile phase A was added. 10 μl of the final extract was injected into the UPLC-MS/MS system.

2.5. Method development and validation

2.5.1. Method development and optimization

2.5.1.1. MS/MS optimization. For each compound, a solution of 1 mg L⁻¹ in methanol was prepared for optimization of the MS/MS parameters by means of an infusion experiment. The individual solutions were directly infused into the MS/MS system at as flow rate of 10 μl min⁻¹, combined with the flow from the UPLC-system by means of a t-junction. UPLC-system was set at a flowrate of 0.4 ml min⁻¹ with a mobile phase composition of 80:20 eluent A:B. S-lens values, collision energies and precursor and product ion masses were optimized for each compound as shown in Table 1.

2.5.1.2. Chromatographic separation. Multiple columns were tested to achieve retention, good chromatographic separation and best possible peak shape for all compounds. Finally, an Acquity UPLC BEH C18 reversed phase column (2.1 × 100 mm, 1.7 μm, Waters) was chosen for separation because of its excellent reproducibility, best possible peak shape, robustness and the ability to perform at very low pH values and high temperatures. A typical chromatogram at LLOQ concentrations is shown in Fig. 1.

2.5.1.3. Sample preparation. To minimize degradation of the photolabile compounds amiodipine and nifedipine, amber colored Eppendorf tubes and vials were used to perform sample preparation. A mixture of methanol and acetonitrile 1:1, containing the internal standard, was used for protein precipitation for optimal miscibility and precipitation of the plasma. After precipitation and vortexing, the samples were placed in the refrigerator to enhance precipitation. After centrifugation, the supernatant was 1:3 diluted with mobile phase A to be compatible with the starting conditions of the chromatographic gradient.

2.5.2. Method validation

The method validation was based on FDA guidelines for bioanalytical validation [27] and includes the following parameters.

2.5.2.1. Linearity. A calibration curve for each analyte was constructed using eight calibration standards and each standard was prepared in duplicate. In each run, a blank plasma sample with IS and a blank plasma sample without IS were also analyzed. The calibration curves were formed using the peak area ratios for the analytes and their corresponding internal standard (response) versus the concentrations applying linear least square regression with a weighing factor of 1/x and excluding of the origin. The correlation coefficient had to be at least 0.995 for all compounds.

2.5.2.2. Limits of quantification. Lower limit of quantitation (LLOQ) was determined by analyzing six replicates of a LLOQ-standard and was defined as the lowest concentration that could be quantified with an accuracy and precision within ± 20%. The upper limit of quantitation (ULOQ) was defined as the highest standard concentration with a precision that does not exceed 15% CV and an accuracy within 15% of the nominal concentration.

2.5.2.3. Accuracy and precision. Intra- and inter-day accuracy and precision were examined by analyzing replicates of QC's at three concentration levels. For intraday precision and accuracy 6 replicates per level were used. For interday precision duplicates of each QC level were analyzed on 6 different days. Precision should not exceed 15% CV and accuracy should be within 15% of the nominal concentration.

2.5.2.4. Stability. Stability of the QC samples was examined by performing the sample preparation and analyzing the extracts 24, 48 & 168 h after storage. Samples were stored in the refrigerator at 2–8 °C during the whole experiment.

Stability of the extracts in the autosampler was examined by analyzing the extracts 24, 48 & 168 h after injecting them for the first time. Extracts were stored in the autosampler at 10 °C during the whole experiment. For both stability experiments, calibration was performed with freshly prepared calibration standard. Measured concentrations of the stored extracts within 15% of the original result (t = 0) were considered acceptable.

Long-term stability of the stock solutions was examined by comparison of two sets of stock solutions. The second set was prepared 9 months after the first one.

Freeze-thaw stability was not studied because this is not a part of our sample routine. Patient-samples are stored in the dark at 2–8 °C after arrival upon analysis.

2.5.2.5. Selectivity, recovery and matrix effects. Recovery and matrix effects were studied according to the method of Matuszewski.

Three sets of samples were prepared:

Set A: Standards in deionized water

Set B: Standards spiked after sample preparation in plasma of 5 different lots

Set C: Standards spiked before sample preparation in plasma of 5 different lots

For each set blanks, QC L and QC H were prepared in duplicate. Selectivity was determined by analyzing blanks of the different plasma lots. One lot consisted of the blank plasma used for preparation of standard and QC's. The other four lots consisted of pooled plasma from 3 or 4 patient samples not using antihypertensive drugs. For all compounds, peak areas less than 20% of the peak area at LLOQ level in the 5 different lots was considered acceptable.

Recoveries (RE) of all analytes were determined by comparing the quantitative results of set B and C (C/B x 100%) for each analyte. Matrix effects (ME) were studied by comparing set A and B. (B/A x 100%)

The efficiency of the total process (PE) is calculated by (C/A x 100%)

2.5.2.6. Carry over. Carry-over was investigated by analyzing blanks after the standards with the highest concentration of the calibration curve. Carry over was expressed as percentage by comparing the measured response of the blank with the response of the highest calibration standard.

3. Results

3.1. Validation

3.1.1. Linearity

The linearity of the method was obtained for all analytes over the calibration range detailed in Table 2. The calculated concentrations of the calibration standards were within the acceptance criteria and the coefficient of correlation (r) was > 0.995 for all compounds. The complete set of eight standards in duplicate was used to construct a calibration curve for all compounds except hydrochlorothiazide, nifedipine and spironolactone. As a result of the lower signal to noise ratios of hydrochlorothiazide, in comparison with the other compounds in this method, the linear range of this compound was limited to the five highest standard levels. For nifedipine and spironolactone only the...
highest calibration level was excluded.

3.1.2. Limits of quantitation
In Table 2 LLOQs and ULOQs are stated for all compounds. Due to the main purpose of this method to assess (non-)adherence when a patient visits the outpatient clinic, the LLOQ should be as low as possible to be able to assess non-adherence at several hours after the last assumed intake, preferably up to 24 h to assess adherence at a random point. The ULOQ is for the same reason of less importance.

3.1.3. Accuracy and precision
Results of intra-day and inter-day precision and accuracy determined at three concentrations are described in Table 2. All analytes except hydrochlorothiazide and spironolactone fulfilled the acceptance criteria since bias and CV did not exceed 20% for LLOQ and 15% for other QC concentrations. For hydrochlorothiazide, the lowest QC level was lower than the achieved LLOQ. The concentration level of QC criteria since bias and CV did not exceed 20% for LLOQ and 15% for all compounds except for amlodipine (20.2%) and hydrochlorothiazide (36%).

3.1.4. Stability
All analytes, except for spironolactone and canrenone, were found stable for 168 h in QC samples stored in the dark at −20°C directly after arrival and subsequently centrifugation to avoid decomposition to canrenone. All analytes were found stable for 168 h in the autosampler at 10°C. Stock solutions are stable for at least 9 months when stored in the dark at −18°C.

3.1.5. Selectivity, recovery and matrix effects
Calculation of matrix effects was based on duplicates of 5 different lots of spiked pooled patient samples for each analyte except amlodipine. One lot was tested positive for amlodipine and therefore was excluded for calculation. Further investigation of the positive lot revealed that one of the plasma samples considered blank was from a patient using amlodipine. After exclusion of the positive plasma sample for amlodipine, measured peak areas for all other samples were less than 20% of the corresponding peak area at LLOQ level for all compounds. The observed matrix-effects presented in Table 3 were between 80 and 120% for all compounds except for amlopril (Low and High QC level) and nifedipine (Low QC level). Recoveries between 80 and 120% were achieved for all compounds except for the low QC level of amlodipine and hydrochlorothiazide. Process efficiencies were between 80 and 120% for all compounds with coefficients of variation less than 4% for QC High. For QC Low CV% were less than 15% for all compounds except amlodipine (20.2%) and hydrochlorothiazide (36%).

3.1.6. Carry over
The carry over, expressed as percentage, of all analytes in blanks analyzed directly after the highest standard of the calibration curve

### Table 2

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Correlation Coefficient $r$</th>
<th>LLOQ (μg/l)</th>
<th>Calibration Range (μg/l)</th>
<th>Concentration QC Levels (μg/l)</th>
<th>Intra-day Accuracy (Bias%)</th>
<th>Precision (CV%)</th>
<th>Inter-day Accuracy (Bias%)</th>
<th>Precision (CV%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>amlodipine</td>
<td>0.9995</td>
<td>0.5</td>
<td>1–400</td>
<td>2.3</td>
<td>2.6</td>
<td>7.6</td>
<td>6</td>
<td>0.4</td>
<td>12.6</td>
</tr>
<tr>
<td>canrenone</td>
<td>0.9984</td>
<td>1</td>
<td>5–2400</td>
<td>13.4</td>
<td>1.8</td>
<td>2.6</td>
<td>6</td>
<td>−0.5</td>
<td>3.8</td>
</tr>
<tr>
<td>enalapril</td>
<td>0.9997</td>
<td>0.2</td>
<td>2–580</td>
<td>2.8</td>
<td>8.9</td>
<td>4.3</td>
<td>6</td>
<td>9.2</td>
<td>3.9</td>
</tr>
<tr>
<td>enalaprilate</td>
<td>0.9997</td>
<td>0.8</td>
<td>1–450</td>
<td>2.6</td>
<td>7.4</td>
<td>7.2</td>
<td>6</td>
<td>6.8</td>
<td>7.2</td>
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<tr>
<td>hydrochlorothiazide</td>
<td>0.9991</td>
<td>40</td>
<td>60–2300</td>
<td>10.9</td>
<td>−32.8</td>
<td>51.7</td>
<td>6</td>
<td>−8.3</td>
<td>49.4</td>
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<td>losartan</td>
<td>0.9997</td>
<td>0.5</td>
<td>4–1900</td>
<td>9.7</td>
<td>−3.2</td>
<td>4.2</td>
<td>6</td>
<td>7.0</td>
<td>4.5</td>
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<tr>
<td>losartan carboxylic acid</td>
<td>0.9999</td>
<td>2</td>
<td>10–5000</td>
<td>38.7</td>
<td>3.7</td>
<td>3.7</td>
<td>6</td>
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<td>2.5</td>
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<td>nifedipine</td>
<td>0.9991</td>
<td>4</td>
<td>5–1100</td>
<td>10.0</td>
<td>2.7</td>
<td>8.8</td>
<td>6</td>
<td>5.0</td>
<td>5.6</td>
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<td>perindopril</td>
<td>0.9996</td>
<td>0.5</td>
<td>1–480</td>
<td>2.5</td>
<td>3.6</td>
<td>3.2</td>
<td>6</td>
<td>2.3</td>
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<td>1–500</td>
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<td>6</td>
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<td>5–1000</td>
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<td>5</td>
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<td>40.0</td>
<td>−7.9</td>
<td>10.1</td>
<td>6</td>
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<td>6.8</td>
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</table>

more evident and utilizes a faster chromatographic separation resulting in a lower plasma sample matrix effect resulting in a process efficiency of 107% at low and high QC level respectively. By combining the actual drug level with that of the active metabolite, sensitivity to detect non-adherence can be increased as compared to only drug levels. Therapeutic ranges for antihypertensive drugs are not available, since due to the wide therapeutic window therapeutic drug monitoring is uncommon. For the purpose of assessment of (non-)adherence, a low LLOQ is required. The LLOQs we found allow reliable assessment up to 24 h after intake of the drug except for hydrochlorothiazide. Since the main purpose of this method will be assessment of (non-)adherence rather than obtaining exact drug levels for titration of treatment, the main characteristic is the lower limit of quantification. The drug level of the drug and the active metabolite can be combined to conclude whether and when the drug has been used. To allow a definite conclusion on drug adherence independent of timing of the last dosage, positive values during 24 h after intake are desirable.

5. Conclusion

The described method provides accurate and sensitive results for monitoring adherence of patients using antihypertensive drugs and is already successfully applied at our hospital. The addition of active metabolites to the method is essential for this purpose, especially for spironolactone which degrades rapidly to canrenone in plasma samples. In this paper, a method of analysis for perindoprilate, the active metabolite of perindopril, is reported for the first time. By combining the analytical results of the drug and the corresponding active metabolite, drug adherence can be monitored up to 24 h after intake for all drugs except hydrochlorothiazide. Analyzing the active metabolites was therefore found essential for accurate classification of drug adherence.

In conclusion, this method is a reliable, quick and easy way to assess drug adherence at relatively low costs compared to electronic monitoring or observed drug intake in the clinic.

Funding

This study was supported by an Erasmus MC Mrace efficacy grant.

Table 3

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Matrix effect (ME)</th>
<th>Recovery (RE)</th>
<th>Process efficiency (PE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>QC low (mean (%)</td>
<td>CV (%)</td>
<td>QC high (mean (%)</td>
</tr>
<tr>
<td>Amlodipine</td>
<td>144 3.6</td>
<td>131 4.7</td>
<td>74 6.9</td>
</tr>
<tr>
<td>Canrenone</td>
<td>100 5.4</td>
<td>102 5.1</td>
<td>84 5.9</td>
</tr>
<tr>
<td>Enalapril</td>
<td>107 2.0</td>
<td>108 1.0</td>
<td>96 3.7</td>
</tr>
<tr>
<td>Enalaprilate</td>
<td>106 8.2</td>
<td>106 4.3</td>
<td>89 5.0</td>
</tr>
<tr>
<td>Hydrochlorothiazide</td>
<td>88 19.1</td>
<td>107 4.6</td>
<td>115 55</td>
</tr>
<tr>
<td>Losartan</td>
<td>106 3.8</td>
<td>108 4.7</td>
<td>95 3.5</td>
</tr>
<tr>
<td>Losartan-carboxylic acid</td>
<td>104 1.9</td>
<td>105 2.8</td>
<td>95 9.9</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>124 4.0</td>
<td>110 1.0</td>
<td>88 9.2</td>
</tr>
<tr>
<td>Perindopril</td>
<td>106 2.7</td>
<td>107 2.3</td>
<td>96 4.0</td>
</tr>
<tr>
<td>Perindopril-late</td>
<td>112 0.6</td>
<td>107 2.7</td>
<td>91 4.1</td>
</tr>
<tr>
<td>Spironolactone</td>
<td>104 12.1</td>
<td>110 4.8</td>
<td>86 6.6</td>
</tr>
<tr>
<td>Valsoartan</td>
<td>98 7.7</td>
<td>109 1.3</td>
<td>98 6.0</td>
</tr>
</tbody>
</table>

Ranged from 0.01% for perindopril to 0.13% for losartan carboxylic acid at the highest.

3.2. Clinical application

In the clinical validation study, at least 6 patients were included for each compound. Both peak and trough levels were measured to investigate the complete analytical range. Trough levels for hydrochlorothiazide, enalapril, losartan, perindopril or spironolactone were below the LLOQ, however for the latter four their corresponding active metabolites were found positive.

4. Discussion

Application of a suitable ultra performance liquid chromatography column combined with mass spectrometry resulted in the development and validation of a high throughput multimethod for antihypertensive drugs by applying a simple extraction protocol followed by a 6 min analytical runtime. In comparison to the multimethod by De Nicolo et al. [25], our method of analysis includes a sample preparation without a time-consuming evaporation step, a lower plasma sample volume and utilizes a faster chromatographic separation resulting in a more efficient routine analysis.

Chromatographic separation and reproducible gaussian peakshape was obtained after extensively testing columns and chromatographic parameters. Raising the column temperature to 70 °C resulted in a significant better peakshape of enalaprilate and perindoprilate but thereby limiting the number of suitable columns. At the same time retention for the early eluting compound hydrochlorothiazide had to be accomplished. Therefore, starting the gradient with a low percentage of organic modifier was applied. The combination of a steep gradient and a mobile phase with a low pH value produced the best result for all compounds. Because of the photolabile properties of amlodipine, nifedipine amber colored Eppendorf tubes and autosampler vials were applied to minimize degradation. This all resulted in excellent linearity, combined with a large concentration range for all compounds.

However, for hydrochlorothiazide a lower signal to noise was determined. This results in a LLOQ concentration above the before estimated value which furthermore exceeds the concentration of QC Low. This explains the poor validation results for QC low. However, QC Medium and High met all the requirements for validation. For spironolactone, the highest calibration level was excluded. However, the achieved ULOQ for spironolactone (1000 μg L⁻¹) is still far above the highest measured concentration in our patient population.

Both intra- and inter-day accuracy exhibit a negative bias for spironolactone and a positive bias for canrenone, most likely caused by degradation of spironolactone to canrenone. For monitoring drug adherence this is not an issue because the result is based on the sum of the two.

The degradation of spironolactone is also the reason for the limited stability of this compound. All other compounds were found stable for 1 week before sample preparation as well as in the final extract.

Measured matrix-effects and recoveries were between 80 and 120% for all compounds except amlodipine.

However, the observed lower recovery for amlodipine is compensated by the higher matrix effect resulting in a process efficiency of 107 and 116% at low and high QC level respectively.

By combining the actual drug level with that of the active metabolite, sensitivity to detect non-adherence can be increased as compared to only drug levels. Therapeutic ranges for antihypertensive drugs are not available, since due to the wide therapeutic window therapeutic drug monitoring is uncommon. For the purpose of assessment of (non-)adherence, a low LLOQ is required. The LLOQs we found allow reliable assessment up to 24 h after intake of the drug except for hydrochlorothiazide. Since the main purpose of this method will be assessment of (non-)adherence rather than obtaining exact drug levels for titration of treatment, the main characteristic is the lower limit of quantification. The drug level of the drug and the active metabolite can be combined to conclude whether and when the drug has been used. To allow a definite conclusion on drug adherence independent of timing of the last dosage, positive values during 24 h after intake are desirable.