Novel contribution to the simultaneous monitoring of pramipexole dihydrochloride monohydrate and levodopa as co-administered drugs in human plasma utilizing UPLC–MS/MS

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Abstract
An efficient, selective, sensitive, and rapid ultra-performance liquid chromatography tandem mass spectrometry method was established and validated for the quantification of pramipexole dihydrochloride monohydrate and levodopa simultaneously in human plasma with the aid of diphenhydramine as an internal standard. A simple protein precipitation technique with HPLC grade acetonitrile was efficiently utilized for the cleanup of plasma. The analysis was performed using a Hypersil gold 50 mm x 2.1 mm (1.9 μm) column and a mobile phase of 0.2% formic acid and methanol (90:10 v/v). The triple-quadrupole mass spectrometer equipped with an electrospray source operated in the positive mode was set up in the selective reaction monitoring mode (SRM) to detect the ion transitions m/z 212.15 → 153.01, m/z 198.10 → 135.16, and m/z 255.75 → 166.16 for pramipexole dihydrochloride monohydrate, levodopa, and diphenhydramine, respectively. The method was thoroughly validated according to FDA guidelines and proved to be linear, accurate, and precise over the range 100–4000 pg/mL for pramipexole dihydrochloride monohydrate and 60–4000 ng/mL for levodopa. The proposed method was effectively applied for monitoring both drugs in plasma samples of healthy volunteers.

Keywords
Pramipexole dihydrochloride monohydrate, levodopa, diphenhydramine, ultra-performance liquid chromatography tandem mass spectrometry, healthy volunteers

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Introduction
Pramipexole dihydrochloride monohydrate (PDM) chemically is (S)-2-amino-4,5,6,7-tetrahydro-6-(propy lamino) benzothiazole dihydrochloride monohydrate1 (Figure 1). PDM is a non-ergot dopamine agonist prescribed for the treatment of Parkinson’s disease. PDM is either used as mono-therapy in early treatment of younger patients or as an adjuvant to levodopa (LVD) therapy in older patients. It is beneficial in reducing “off” periods with LVD and in ameliorating fluctuations in mobility in the later stages of the disease.2 Several analytical techniques were established to quantify PDM as a single component, including amperometry,3 voltammetry,4 spectrophotometry,5–7 high performance thin layer chromatography (HPTLC),8 gas chromatography mass spectrometry (GC-MS),9 high performance liquid chromatography (HPLC),10–14 liquid chromatography tandem mass spectrometry (LC–MS/MS),15–22 and capillary electrophoresis (CE).23,24

LVD chemically is 3-hydroxyl-L-tyrosine1 (Figure 1). LVD is still the most effective and the first drug of choice for treating Parkinson’s disease. It is converted by decarboxylation into dopamine, nevertheless, unlike dopamine; LVD has the ability to penetrate the blood–brain barrier and supply a source of dopamine to the

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brain. Long-term treatment with LVD is frequently accompanied with development of various types of undesired motor complications after nearly 2 years, so other classes of antiparkinsonism drugs such as dopamine agonists (e.g., PDM), catechol-O-methyl transferase inhibitors, or monoamine oxidase type B inhibitors should be used as adjuvant therapy. Literature survey has revealed several methods for the quantification of LVD such as, ¹H nuclear magnetic resonance, spectrophotometry, fluorimetry, chemometry, HPLC, TLC, electrochemistry, LC–MS/MS, and CE.

Combination treatment of LVD with PDM was used for the treatment of Parkinson’s disease. Thus, it was of utmost importance that the plasma concentration of LVD is determined in the patients treated with PDM. However, development of such method was very challenging; as the small doses of PDM in humans has required a sensitive and selective method for measuring plasma levels especially when administered with other high dose one like LVD. Hitherto, there is no reported method in the literature dealing with the simultaneous determination of PDM and LVD as two drugs of different classes which may possibly be co-administered in many therapeutic cases. Thus, the objective of the present study was to develop and validate an accurate, efficient, reliable, economical, and sensitive LC–MS/MS method for the quantitative determination of PDM and LVD simultaneously in human plasma. This approach would be highly beneficial for the generation of clinical data than separate assays. In addition to accomplish a comparative study between the developed HPLC–MS/MS methods for the determination of PDM and LVD as single components and our method regarding the extraction technique and the chromatographic conditions in order to shed the light on the privileges presented by our method.

**Experimental**

**Materials and reagents**

PDM reference standard material was kindly supplied by EVA PHARM, Co., Cairo, Egypt. The purity of PDM was found to be 99.29% according to a reported method, while LVD and diphenhydramine (DPN) reference standard materials were kindly supplied by Sigma Pharmaceutical Industries, Steinheim, Germany. The purity of LVD was found to be 99.05% according to a reported method. Ramixole tablets nominally containing 0.25 mg of PDM per tablet, B.N. 212233 (EvaPharma, Cairo, Egypt). Sinemet tablets nominally containing 250 mg of LVD and 25 mg of carbidopa per tablet B.N. K023077 (manufactured by Merck Sharp & Dohme—Italy and packed by Global napi Pharmaceutical—Egypt). Ramixole and Sinemet tablets were purchased from local market.

All solvents and materials were of HPLC grade. Methanol (Fischer Scientific UK Ltd, Loughborough, UK), acetonitrile, ammonium acetate and ammonium formate (Merck, Darmstadt, Germany) and deionized water (Purelab flex, ELGA) were used. Human plasma was purchased from Vaccera (Giza, Egypt), kept frozen until used after gentle thawing.

**Instruments**

Quantitative analysis was accomplished on a TSQ Quantum Access MAX triple stage quadrupole mass spectrometer, Thermo Scientific, New York, USA, equipped with electrospray ionization (ESI) operated in the positive ionization mode. Chromatographic separation was carried out using Accela UPLC system; composed of Accela 1250 quaternary pump and Accela open autosampler, New York, USA (operated at 25°C). Xcalibur software version 2.2 was used to control all parameters of UPLC, MS, and analysis of the obtained data.

**Mass spectrometric conditions**

Positive-ion mass spectrometric detection method with ESI and selected reaction monitoring (SRM) mode was used. The optimized parameters were: turbo ion spray temperature of 400°C, capillary temperature of 270°C, auxiliary gas of 2 psi, sheath gas of 20 psi, ion spray voltage of 3500 V, and capillary offset of 35. The quadrupole mass spectrometer operated at the SRM mode was utilized for the monitoring of the transitions of molecular ions to the product ions for PDM m/z 212.15→153.01, LVD 198.10→135.16, and DPN (IS) 255.75→166.16 using the collision energies of 12, 18, and 14 eV for the three analytes, respectively.
Chromatographic conditions

Chromatographic separation of the analytes was carried out on Hypersil Gold column (C18 bonded ultra-pure silica based column) 50 mm × 2.0 mm (1.9 μm) from Thermoscientific, New York, USA. Isocratic elution was performed at room temperature using freshly prepared mobile phase of 0.2% formic acid aqueous solution: methanol (90: 10 v/v) at a flow rate of 0.25 mL/min.

Procedures

Preparation of stock and working standard solutions

Stock standard solutions. 100.0 μg/mL of PDM, and DPN (IS) was prepared separately in methanol, while 100.0 μg/mL of LVD was prepared in deionized water.

Working standard solutions. Accurate aliquots were transferred from the corresponding stock standard solutions with appropriate dilution into 10-mL volumetric flasks with methanol. All stock standard solutions were stored in the dark at 4°C and were found to be stable for more than 15 days.

Preparation of calibration standards and quality control samples

Aliquots of 800 μL of blank human plasma were spiked with 100 μL of increasing concentrations of each of PDM and LVD from their working standard solutions. The final concentrations in the plasma samples were 100.0, 200.0, 500.0, 1000.0, 2000.0, and 4000.0 pg/mL for PDM and 60.0, 100.0, 500.0, 1000.0, 2000.0, and 4000.0 ng/mL for LVD as illustrated in Table 1. The plasma calibration curve consisted of a blank sample (matrix sample processed in absence of the internal standard), a zero sample (matrix sample processed in presence of the internal standard), and six nonzero samples covering the expected range, including lower limit of quantification (LLOQ).

Similarly, quality control standards (QCs) used in the validation were prepared for the practically determined LLOQ with final concentration of 100 pg/mL and 60 ng/mL, low quality control (LQC) with final concentration of 250 pg/mL and 200 ng/mL, medium quality control (MQC) with final concentration of 1500 pg/mL and 1500 ng/mL and high quality control (HQC) with final concentration of 3500 pg/mL and 3000 ng/mL for each of PDM and LVD, respectively.

Preparation of samples

Plasma samples stored at −70°C were thawed at room temperature on the day of analysis.

An aliquot of 10 μL was transferred from DPN (IS) working solution (10 μg/mL) which is equivalent to 100 ng/mL (final volume) and was added to 1 mL plasma. Precipitation technique was applied, where 2 mL of acetonitrile were added to the spiked plasma sample, vortexed for 20 s, and centrifuged (at 3500 r/min) for 30 min. The upper clear supernatant was accurately transferred into dry clean injection vials after filtration through a 0.45 μm membrane filter. Finally, 10 μL of this solution was injected into the UPLC–MS/MS system. The peaks were interpreted based on the reported peak areas. Concentrations of PDM and LVD in unknown samples were calculated by referring to the prepared calibration curves.

Method validation

Specificity

The specificity of the method was demonstrated by examining different batches of blank human plasma to indicate the absence of chromatographic interference from endogenous plasma components.

Calibration curve

The calibration spiked plasma samples curves were prepared as mentioned under “Preparation of calibration standards and quality control samples.” The curves were constructed by plotting the peak area ratios of each analyte/IS against the corresponding concentrations. The results are considered acceptable when the correlation coefficient (r) of the calibration curve is less than 0.99 and the back-calculated concentrations of each point is within ±15% from the nominal value except at the LLOQ where deviation is permitted up to ±20%.

Precision and accuracy

Inter- and intra-assay precision and accuracy were validated by investigating six replicates at the LLOQ

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Table 1. Preparation of calibration standards and quality control samples for pramipexole and levodopa.

<table>
<thead>
<tr>
<th>Plasma volume (μL)</th>
<th>Adding 100 μL of each working standard solution (μg/mL)</th>
<th>Final volume (μL)</th>
<th>Final plasma concentration (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>800</td>
<td>PDM 600, LVD 100</td>
<td>1000</td>
<td>PDM 100, LVD 60</td>
</tr>
<tr>
<td></td>
<td>2 PDM 1000, LVD</td>
<td></td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>5 PDM 5000, LVD</td>
<td></td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>10 PDM 10,000, LVD</td>
<td></td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td>20 PDM 20,000, LVD</td>
<td></td>
<td>2000</td>
</tr>
<tr>
<td></td>
<td>40 PDM 40,000, LVD</td>
<td></td>
<td>4000</td>
</tr>
<tr>
<td></td>
<td>LQC 2.5 PDM 2000, LVD 200</td>
<td></td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>MQC 15 PDM 15,000, LVD 1500</td>
<td></td>
<td>1500</td>
</tr>
<tr>
<td></td>
<td>HQC 35 PDM 30,000, LVD 3500</td>
<td></td>
<td>3500</td>
</tr>
</tbody>
</table>
together with the three different QC levels as previously described on different days and on the same day, respectively. Intra- and inter-day precision were evaluated by the relative standard deviation (RSD %) at each QC level. However, accuracy was estimated by calculating the difference between the calculated and theoretical concentrations (relative error, RE %). For satisfactory results, variation should be ±15%, except for LLOQ where variation was allowable to 20%.

Recovery

The recovery for PDM and LVD was estimated through comparing their responses from preextracted replicate QC samples (low, medium, and high) with their responses from postextracted plasma standard sample (i.e., spiking is performed after plasma extraction) at equivalent concentrations.

Matrix effects

The evaluation of the effect of plasma components on the ionization of the analytes and the IS was performed through the comparison of the responses of the postextracted plasma standard QC samples (n = 4) with the response of analytes from neat samples at equivalent concentrations.

Stability experiments

Short term stability: A triplicate of LQC, MQC, and HQC samples was examined by thawing these samples at room temperature and then keeping them for a period of 6 h before analysis. Freeze-thaw stability was performed over three freeze-thaw cycles by thawing a triplicate of frozen LQC, MQC, and HQC samples at room temperature for more than 2 h and then freezing them at −70°C overnight for each cycle.

Post-preparative stability: a triplicate of LQC, MQC, and HQC samples were processed, analyzed, and left in the autosampler at 25°C for 1 day.

Long term stability: a triplicate of LQC, MQC, and HQC samples were stored at −70°C and analyzed at the end of the study (20 days).

Stock solutions stability: (0.1 mg/mL of each analyte and IS) was evaluated after 15 days of storage in a refrigerator (4°C). The stored solutions were diluted to obtain the same concentration levels as the short-term stability assay.

Under all the previously stated conditions, the stability was estimated by comparing the mean recovery of the analytes and IS achieved from stored samples to the mean values achieved from freshly prepared sample, at the same concentration levels.

Application to biological samples

After being fully validated, the proposed method was applied for the quantification of PDM and LVD in plasma samples of six healthy volunteers. The volunteers received a single oral dose of Sinemet® tablet containing 250 mg of LVD and Ramixole® tablet containing 0.25 mg PDM under fasting conditions. Blood samples of 5 mL were withdrawn from each volunteer and transferred into amber tubes according to the following time schedule: 0.0, 0.15, 0.3, 0.45, 1.5, 2.5, 4.0, and 5.0 h after drug administration, using ethylenediaminetetraacetic acid as anticoagulant. The samples were centrifuged immediately at 3500 r/min for 10 min to obtain the plasma. The plasma samples were labeled and kept frozen at −70°C until being analyzed.

Results

Method development

Optimization of the sample preparation is critical in order to efficiently quantify the studied drugs in human plasma. Therefore, different precipitation solvents using methanol and acetonitrile were tried for the simultaneous determination of PDM and LVD. Best results were attained using 2 mL acetonitrile as a precipitating solvent. Consequently, the mass spectrometric parameters and the chromatographic conditions were thoroughly optimized.

With respect to the mass spectrometric parameters, 1.00 μg/mL of the neat solutions of each analyte were directly infused into the mass spectrometer with the aim of adjusting the precursor and product ions. The ions were scanned in a mass range of 100–500 m/z. As PDM, LVD, and DPN (IS), are protonated under the utilized acidic chromatographic conditions, accordingly, the mass spectrometer was run in the positive polarity mode utilizing ESI technique which has resulted in the ideal intensity for the analytes’ precursor ions and product ions.

The Q1 full-scan mass spectra of PDM, LVD, and DPN (IS) displayed the protonated molecular ions [M+H]+ 212.15, 198.10, and 255.75 for the three analytes, respectively. Characteristic ions were created in the Q2 resulting from using appropriate collision activated dissociation gas and collision energy, where the following MS/MS transitions were selected 212.15 → 153.01 for PDM, 198.10 → 135.16 for LVD, and 255.75 → 166.16 for DPM (IS) as demonstrated in Figure 2. Moreover, optimization of both the capillary temperature and sheath gas flow was established as both of them can considerably alter the sensitivity. As a consequence, the intensity of the analytes was enhanced upon adjusting the capillary temperature at 270°C and sheath gas at 20 psi. On the other hand, slight
alterations in ion spray voltage have not displayed any recognizable effect on the signal intensity and it was maintained at 3500 V.

Concerning the chromatographic conditions, several parameters were inspected as the organic modifier, pH, organic modifier-aqueous ratio and the column. It was clear that mobile phase composition had a significant effect on the selectivity and sensitivity of separation. Acetonitrile and methanol were employed as the organic modifiers besides several mobile phase additives were tested as 0.2% aqueous formic acid, ammonium formate and ammonium acetate in varying pH.

Figure 2. Product ion spectra of [M + H]$^+$ of pramipexole dihydrochloride monohydrate, (b) levodopa, and (c) diphenhydramine (IS).
values from 2.5 to 7.0. The best sensitivity was accomplished using 0.2% aqueous formic acid and methanol. Also, different ratios of methanol: 0.2% formic acid (pH 2.5) were examined where methanol: 0.2% formic acid aqueous solution (10: 90 v/v) was selected. Formic acid solution has aided in achieving good response for MS detection operating in the positive mode. The optimized conditions have led to the best peak shape,
intensity and resolution, in addition all the analytes were eluted within a narrow retention time range (0.75–0.83 min) as demonstrated in Figure 3. The reproducibility of retention times for the analytes, was acceptable for about 50 injections on the same column. Regarding the columns used, two columns Hypersil-Gold and Bio Basic-8 were tested in order to achieve the best chromatographic performance. Hypersil-Gold column which was maintained at room temperature has displayed the better response even at LLOQ levels of the analytes.

Method validation

A full validation process was conducted as follows according to Guidance for Industry Bioanalytical Method Validation, recommended by FDA.44,45

Specificity

By comparing the chromatograms of blank plasma and blank plasma spiked with PDM or LVD at the LLOQ; no interfering peaks was observed from the endogenous plasma constituents at the same retention times of the analytes or the IS.

Calibration curve

The constructed plasma calibration curves were linear in the range of 100–4000 pg/mL for PDM and 60–4000 ng/mL for LVD with good reproducibility. The correlation coefficients of the all curves were higher than 0.999.

### Table 2

<table>
<thead>
<tr>
<th>QC level</th>
<th>Pramipexole</th>
<th>Levodopa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intraday ($n = 6 \times 3$)</td>
<td>Intraday ($n = 6$)</td>
</tr>
<tr>
<td>LLOQ</td>
<td>RE%</td>
<td>RSD%</td>
</tr>
<tr>
<td>LQC</td>
<td>-5.9</td>
<td>1.72</td>
</tr>
<tr>
<td>MQC</td>
<td>-6.9</td>
<td>1.43</td>
</tr>
<tr>
<td>HQC</td>
<td>7.00</td>
<td>1.67</td>
</tr>
<tr>
<td></td>
<td>-3.94</td>
<td>2.23</td>
</tr>
</tbody>
</table>

% RSD: percent relative standard deviation; % RE: percent relative error ($n = 3$ days, 6 replicates per day).

### Table 3

<table>
<thead>
<tr>
<th>Stability condition</th>
<th>QC level</th>
<th>Precision (RSD, %)</th>
<th>Accuracy (RE, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PXL</td>
<td>LVD</td>
</tr>
<tr>
<td>Short term stability</td>
<td>LQC</td>
<td>0.43</td>
<td>2.53</td>
</tr>
<tr>
<td></td>
<td>MQC</td>
<td>0.20</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>HQC</td>
<td>0.37</td>
<td>0.15</td>
</tr>
<tr>
<td>Freeze-thaw stability</td>
<td>LQC</td>
<td>1.40</td>
<td>2.61</td>
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<tr>
<td></td>
<td>MQC</td>
<td>1.11</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>HQC</td>
<td>0.24</td>
<td>0.30</td>
</tr>
<tr>
<td>Postoperative stability</td>
<td>LQC</td>
<td>0.77</td>
<td>2.17</td>
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<tr>
<td></td>
<td>MQC</td>
<td>0.20</td>
<td>0.68</td>
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<tr>
<td></td>
<td>HQC</td>
<td>0.64</td>
<td>0.12</td>
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<tr>
<td>Long term stability</td>
<td>LQC</td>
<td>1.31</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>MQC</td>
<td>0.17</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>HQC</td>
<td>0.22</td>
<td>0.82</td>
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<tr>
<td>Stock solution stability</td>
<td>LQC</td>
<td>0.65</td>
<td>2.62</td>
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<tr>
<td></td>
<td>MQC</td>
<td>0.84</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>HQC</td>
<td>0.77</td>
<td>0.17</td>
</tr>
</tbody>
</table>

% RSD: percent relative standard deviation; % RE: percent relative error.

Figure 4. Mean plasma concentration after a single oral dose of (a) 250 mg levodopa and (b) 0.25 mg pramipexole dihydrochloride monohydrate administered to six healthy volunteers.
where: \( y = 0.0036x - 0.0151, R = 0.9991 \) for PDM
\( y = 0.0055x + 0.2228, R = 0.9995 \) for LVD

All the back-calculated concentrations for the points on the calibration curve were within ±15% of the nominal concentrations. The LLOQ was identified as the lowest concentration at S/N ratios of 10 and with RSD < 20%, and was established to be 100 pg/mL for PDM and 60 ng/mL for LVD.

**Precision and accuracy**

Accuracy and precision results were acceptable as they were within ±20% for the LLOQ and ±15% for the other QC samples. The intraday precision (RSD %) varied from 0.42% to 1.22% for PDM and from 0.19% to 2.60% for LVD. While, the intraday accuracy (RE %) was in the range of –7.80% to 3.94% for PDM and from –2.47% to –8.77% for LVD. The interday variability was estimated by examining the QC samples for three successive days. The interday precision (RSD %) ranged from 1.43% to 2.23% for PDM and 0.12% to 2.78% for LVD and the interday accuracy (RE %) was in the range of –3.94% to 7.00% for PDM and from –2.49% to –8.97% for LVD as shown in Table 2.

**Recovery**

The mean extraction recoveries for PDM and LVD were calculated at the QC levels and were found higher than 85%. It varied from 91.62% to 99.84%, for PDM and from 87.44% to 104.49% for LVD.

**Matrix effect**

The matrix effect was calculated through the comparison of the responses obtained from the post extracted plasma QC samples with those of the analytes from neat samples and were found to be in the range of

<table>
<thead>
<tr>
<th>Extraction technique</th>
<th>Mobile phase</th>
<th>Flow rate</th>
<th>Column</th>
<th>Run time</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein precipitation using acetonitrile containing the derivatizing reagent (flourescamine)</td>
<td>Mobile phase (A) consisted of 5 mM ammonium formate: formic acid at 1000: 1 (v/v) and mobile phase (B) was acetonitrile and mobile phase B was acetone. The UHPLC system was held at 25% B for 0.2 min followed by a linear gradient from 25% B to 50% B in 1.8 min</td>
<td>0.5 mL/min</td>
<td>BEH C18 (50 mm × 2.1 mm, 1.7 μm)</td>
<td>3.5 min</td>
<td>42</td>
</tr>
<tr>
<td>Protein plasma precipitation with perchloric acid</td>
<td>Methanol: water (90:10, v/v) containing formic acid 0.5% v/v. Flow rate: 1 mL/min</td>
<td>1 mL/min</td>
<td>Pursuit® C18 (150 mm × 4.6 mm, 5 μm)</td>
<td>6.0 min</td>
<td>41</td>
</tr>
<tr>
<td>Protein plasma precipitation with perchloric acid</td>
<td>0.2% formic acid: acetonitrile (90:10, v/v)</td>
<td>0.2 mL/min</td>
<td>ACE C18 (50 × 4.6 mm, 5 μm)</td>
<td>6.0 min</td>
<td>40</td>
</tr>
<tr>
<td>Protein plasma precipitation with perchloric acid</td>
<td>Methanol: water (10:90, v/v) containing 0.5% formic acid</td>
<td>0.2 mL/min</td>
<td>COSMOSIL 5 C18MS (2 mm I.D. × 150 mm, 5 μm)</td>
<td>0–2 min</td>
<td>39</td>
</tr>
<tr>
<td>Liquid-liquid extraction</td>
<td>10 mM ammonium acetate: methanol (30:70, v/v)</td>
<td>1.2 mL/min</td>
<td>LiChrospher RP-select B (100 mm × 4.0 mm, 5 μm)</td>
<td>3.5 min</td>
<td>15</td>
</tr>
<tr>
<td>Liquid-liquid extraction</td>
<td>0.01 M ammonium acetate buffer (pH 4.4): acetonitrile (30:70, v/v)</td>
<td>0.5 mL/min</td>
<td>CN based column, Thermo Electron Corporation (50 mm × 4.6 mm, 5 μm)</td>
<td>3.0 min</td>
<td>16</td>
</tr>
<tr>
<td>Liquid-liquid extraction</td>
<td>10 mM ammonium formate (pH 7.5): acetonitrile (15:85, v/v)</td>
<td>0.5 mL/min</td>
<td>BEH C18 (100 mm × 2.1 mm, 1.7 μm)</td>
<td>1.5 min</td>
<td>17</td>
</tr>
<tr>
<td>Liquid-liquid extraction</td>
<td>Acetonitrile: 10 mmol ammonium acetate: formic acid (60:40:0.1, v/v/v)</td>
<td></td>
<td>Venusil ASB-C18 (150 mm × 4.6 mm, 5 μm)</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Solid-phase extraction</td>
<td>Acetonitrile: ammonium formate (65:35, v/v),</td>
<td></td>
<td>Hypersil gold</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>The drug extracted from dried blood spot using methanol</td>
<td>10 mM ammonium acetate: methanol (50:50, v/v)</td>
<td>1.0 mL/min</td>
<td>Synergy polar-RP (250 mm × 4.6 mm, 4 μm)</td>
<td>5.1 min</td>
<td>20</td>
</tr>
<tr>
<td>Liquid-liquid extraction</td>
<td>10 mM ammonium formate: acetonitrile (60:40 v/v)</td>
<td>0.5 mL/min</td>
<td>Zorbax SB-C18 (150 mm × 4.6 mm, 3.5 μm)</td>
<td>1.34 min</td>
<td>21</td>
</tr>
<tr>
<td>Protein precipitation coupled with solid phase extraction</td>
<td>5 mM ammonium formate (pH 5.0): acetonitrile (30:70, v/v)</td>
<td>0.5 mL/min</td>
<td>WelchUltimate® XB-CN (100 mm × 2.1 mm, 5.0 μm)</td>
<td>3.0 min.</td>
<td>22</td>
</tr>
</tbody>
</table>

**Table 4.** Comparison of analytical methods developed for the determination of parmipexole and levodopa in human plasma.
93.55–99.67% for PDM and 98.33–105.25% for LVD which proves that the plasma components have not affected the ionization of the analytes.

**Stability experiments**

For all stability experiments, three sets of LQC, MQC, and HQC were handled as previously described in the “Stability experiments” section. The samples were found stable under all the studied conditions, as there was no observed significant difference for each concentration compared with nominal concentration (Table 3).

**Application to biological samples**

The validated UPLC–MS/MS method was effectively applied for the quantification of the co-administered PDM and LVD simultaneously in human plasma. Figure 4 shows the mean plasma concentration versus time curves for the studied drugs. The mean value of T<sub>max</sub> was found to be 2.5 h while the T<sub>1/2</sub> was 4.5 h and the C<sub>max</sub> was 410 pg/mL for PMD while those for LVD were found to be T<sub>max</sub> 1.5 h, the T<sub>1/2</sub> was 3.2 h and the C<sub>max</sub> was 1850 ng/mL. The obtained results are in good agreement with the pharmacokinetic profile of LVD<sup>46</sup> and PDM<sup>16</sup> concentrations in human plasma.

**Discussion**

In spite that several HPLC–MS/MS methods were performed for the determination of PDM<sup>15–22</sup> and LVD<sup>39–42</sup> singly in human plasma, yet, our proposed method is the first performed attempt for their simultaneous determination as was indicated from a thorough literature survey. The extraction technique and the chromatographic conditions of these reported methods are summarized in Table 4. From the data demonstrated in Table 4, it is obvious that our method has demonstrated most of the advantages which could be introduced by an UPLC–MS/MS method. The utilization of the UPLC technique with small column size (50.0 mm × 2.1 mm, 1.9 μm particle size) has resulted in the fastest elution of the analytes among all methods (about 1 min) with good resolution owing to the small particle size. The flow rate of 250 μL/min has resulted in less solvent consumption, thus, the method could be considered as cost effective. Moreover, a simple isocratic mobile phase was established where only 10% organic modifier was used and avoided the use of buffer. Additionally, our sample preparation technique was very simple utilizing acetonitrile for protein precipitation, thus we avoided the sophisticated processes of liquid–liquid extraction or solid phase extraction.

**Conclusion**

A sensitive, selective, and rapid UPLC–MS/MS method was established and validated for quantification of the co-administered drugs; PDM and LVD simultaneously in human plasma. The main benefits obtained from the method are analyzing the drugs of interest on a single chromatographic system with pronounced sensitivity, little solvent consumption, and a short run-time. The obtained results have assured the applicability of the method for the quantification of the analytes in very low levels (pictogram or nanogram concentrations) as that present in human plasma especially when the available resources are limited. The proposed method has allowed for the effective determination of the low dosing drug PDM in human plasma either alone or in combination with LVD as co-administered drugs.

**Declaration of conflicting interests**

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