Determination of venlafaxine and O-desmethylvenlafaxine in dried blood spots for TDM purposes, using LC-MS/MS

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ABSTRACT

Introduction: Dried blood spot (DBS) sampling and quantitative analyses of many current TDM guided drugs is advantageous because of the minimal invasive sampling strategy. Here, a fast and robust liquid chromatography-tandem mass spectrometry method was developed and analytically validated for simultaneous determination of venlafaxine (VEN) and O-desmethylvenlafaxine (ODV) in DBS.

Methods: Six mm circles were punched out from DBS collected on Whatman DMPK-C paper and the DBS was extracted with acetonitrile: methanol 1:3. The total run time was 4.8 min.

Results: The assay was linear in the range 20-1000 µg/L for both VEN and ODV. Assay accuracy and precision was well within limits of acceptation (LLOQ = 20 µg/L). Normal hematocrit concentrations (0.30-0.50) did not influence the results neither did a normal spot volume (40-80 µL). Punch position at the perimeter instead of the center of the blood spot gave a bias ranging from 2.4-10.4%. Correlation between plasma and spiked DBS samples was high. The concentrations found in spiked DBS samples were higher than those in plasma indicating that a conversion factor for translation of DBS to plasma values is needed.

Conclusion: This analytically validated method is suitable for determination of VEN and ODV in DBS and applicable for TDM. The method will be used for TDM of VEN in the Dutch CYSCE multicenter trial (NCT01778907).
3.2.1. INTRODUCTION

Venlafaxine (VEN) is a commonly prescribed antidepressant which is also prescribed and registered for treatment of anxiety and panic disorders. Like several other antidepressants, VEN is metabolized by the liver enzyme Cytochrome P450 2D6 and 2C19 into several metabolites including its main active metabolite O-desmethylvenlafaxine (ODV). The activity of these enzymes is influenced by genetic differences which can cause large inter-individual variation in the drug metabolism and clearance. Additionally, especially in the elderly, plasma concentrations of VEN and ODV may increase substantially, because of aging and the use of CYP 2D6 inhibiting co-medication. Therapeutic drug monitoring is therefore advised.

Several methods for simultaneous determination of VEN and OVD in plasma by liquid chromatography-tandem mass spectrometry (LC-MS/MS) have been reported [1-4]. However, no dried blood spot (DSB) assay has previously been described. DBS sampling is beneficial, because it is a minimal invasive sampling strategy. The blood is obtained by a fingerprick instead of venous puncturing. Additionally, DBS samples can be sent to a laboratory by standard postal service, because the dried sampling cards entail no biohazard risks [5]. These advantages allow patients or their caregivers to collect a blood sample at home. For depressed or other mentally ill patients this can be an important improvement.

Here we report on a novel, validated, and simple DBS assay, for TDM of VEN and ODV, using LC-MS/MS. Method validation included estimation of the correlation between spiked plasma and spiked DBS samples.

3.2.2. MATERIAL AND METHODS

Chemicals and reagents
VEN, ODV, both >99%, were purchased from Wyeth pharmaceuticals (Hoofddorp, the Netherlands) Promazine (IS) was purchased from Sigma Aldrich (Zwijndrecht, the Netherlands). Methanol (Lichrosolv), ammonium acetate (p.a.), acetic acid 100% (p.a.) and trifluoro-acetic acid anhydride (TFAA, GC grade.) were purchased from Merck (Darmstadt, Germany). Acetonitrile and purified water (ULC/MS grade) were purchased from Biosolve BV (Valkenswaard, the Netherlands). Whatman FTK DMPK-C blood sampling cards were purchased from GE Healthcare (Hoevelaken, the Netherlands). Blank EDTA whole blood was obtained from healthy volunteers. The hematocrit (Htc) of this blood was 0.41.
Instrumentation

The LC-MS/MS system consisted of an Accela autosampler and UHPLC Accela pump connected with a TSQ Quantum Acces tandem mass spectrometer with an electrospray ionization source, obtained from Thermo Scientific (Thermo, Breda The Netherlands). System control, data acquisition, and data processing were performed by Excalibur 2.0 software.

Chromatography and Mass spectrometry conditions

A Hypurity Aquastar column, 50×2.1 mm, particle size 5 μm (Thermo, Breda, The Netherlands) was used for chromatographic separation. Mobile phase A consisted of 2 mL TFAA, 35 mL acetic acid 100%, and 5 g ammonium acetate in 1000 mL purified water (pH 3.5). Mobile phase B, was purified water, and mobile phase C consisted out of acetonitrile. The mobile phase ratio was 5% of A and 95% of B during two minutes. After two minutes, the mobile phase ratio changed to 5% of A and 95% of C in a linear slope over one minute. After three minutes, the mobile phase ratio returned to the starting position, and the column was allowed to equilibrate during one minute. The total runtime was 4.8 minutes. The flow rate was kept constant at 0.3 ml/min. Retention times of VEN, ODV, and IS are given in table 1. No carry over was observed for either VEN or ODV (−0.18, −0.72%).

Ionization was achieved in the positive electrospray mode. Spray voltage was 3000 V. Sheath and auxiliary gas pressure were set to respectively, 40 and 10 (arbitrary units). Capillary temperature was 375 ºC, and the collision gas (argon) pressure was 1.5 mTorr. The scan modus was set to selective reaction monitoring (SRM) and the different mass transitions, with their respective lens and collision voltages, which were monitored, are showed in Table 1.

Table 1. LC-MS/MS conditions

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
<th>Parent mass (m/z)</th>
<th>Daughter mass (m/z)</th>
<th>Tube Lens Voltage (V)</th>
<th>Collision Energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEN</td>
<td>2.10</td>
<td>278.2</td>
<td>121.2</td>
<td>75</td>
<td>28</td>
</tr>
<tr>
<td>ODV</td>
<td>1.84</td>
<td>264.2</td>
<td>107.3</td>
<td>74</td>
<td>31</td>
</tr>
<tr>
<td>PMZ (IS)</td>
<td>2.42</td>
<td>285.1</td>
<td>86.4</td>
<td>56</td>
<td>16</td>
</tr>
</tbody>
</table>

Sample preparation

Stock solutions of VEN and ODV with a concentration of 1g/L were prepared in methanol. From both stock solutions 2.5 mL aliquots were mixed and diluted to a working solution with NaCl 0.9%. Blank EDTA whole blood was spiked with the working solution and completed to the same end volumes with NaCl 0.9%, to obtain calibration sample concentrations of 20, 50, 100, 250, 500, and 1000 µg/L respectively. QC samples were
spiked to obtain concentrations of respectively, 20 µg/L = LLOQ, 80 µg/L = QC Low, 300 µg/L = QC medium, and 750 µg/L = QC high. Fifty microliter of the spiked blood was spotted on DMK-C cards and dried overnight at 20-25 °C. With a pipet one bloodspot was carefully made by spotting two small droplets of blood on to the paper. Dried DBS cards were stored at 2-8°C together with a desiccant (silica) bag in a sealed plastic bag. VEN is known to be stable in aqueous solutions and biological matrices for 57 days at 20°C [6]. Therefore stability of a dry DBS cards at 2-8°C was assumed to be at least 2 months. During method development, DBS punches of six millimetre were extracted with different ratios of acetonitrile and methanol. A ratio of 1:3 gave the best recovery results and was therefore used as the extraction fluid. For extraction, 250 µL of extraction fluid containing the IS (final concentration 40 µg/L) was added followed by briefly vortexing and five minutes of shaking. The supernatant was transferred into vials and a volume of 5 µL was injected into the LC-MS/MS.

Method validation

Validation was performed according to the guidelines of the FDA for bioanalytical method validation. To assess matrix effects, blank blood spots from six different volunteers were extracted. Concentrations of VEN and ODV equal to the calibration curve were prepared in the blank extracts and analyzed. The individual slopes of the analyte/IS ratio calibration lines from the different matrices were compared to the slope of a calibration line from the matrix free standard solution. The relative difference between slopes was calculated as the matrix effect. If these differences were <10% no matrix effect was assumed. Additionally, the influence of Htc (ranging from 0.25-0.50 L/L), blood spot volume (ranging from 20-100 µL), and the punch position of the DBS on the assay was validated [7, 8]. Depending on the matrix which is examined, measured drug concentrations may differ due to variable binding to cells and proteins [9]. To assess whether there were any differences between plasma and DBS concentrations, 15 samples with spiked concentrations (ranging from 20-1100 µg/L) of VEN or ODV were analyzed by our plasma and DBS assay. Correlation between the two different methods was analyzed by Pearson’s correlation coefficient and Passing-Bablok regression. Analysis were performed in XLSTAT 2013.4.05.

3.2.3. RESULTS

Six chromatograms from blank DBS of volunteers were visually inspected and no interfering peaks were observed at the ODV and VEN positions (figure 1). The highest co-eluting peaks which were found were 1.7, 2.0, and 0.2% for VEN, ODV, and IS, respectively. Matrix interference caused a substantial positive bias for both VEN (14.7%) and ODV (18.4%).
However, bias was consistent between the individual samples for both VEN (CV: 3.8%) and ODV (CV 5.6%). The assay was found linear over the tested concentration range (r-squared > 0.992), no significant lack of fit was found. Recovery of ODV for the QC low samples was 67%, which is somewhat low. Recovery of all other QC samples was > 80%. The stability of VEN and ODV was not affected in the auto sampler up to at least 48 h. After a period of 6 months storage at 2-8 degrees Celsius, no decline of QC samples was observed. The accuracy and precision was high and at all QC levels the CV and the bias was well within the limits of the FDA (table 2).

Analytical bias related to changes in Hct is shown in figure 2. A decrease in Hct was associated with a negative bias for both VEN and ODV. A Hct of ≤ 0.25 resulted in ≥ 15% bias for both VEN and ODV. The punch position appeared to influence the measured concentration. If the punch was made at the perimeter measured concentrations were higher. Analytical bias of the punches at the perimeter was 8.5 and 4.8% (ratio center/parameter: 0.92 and 0.95) for the QC low and high levels of VEN, respectively. For ODV this was 10.4 and 2.4% (ratio center/parameter: 0.91 and 0.98) at QC low and high level, respectively. The punches from the center were closest to the theoretical concentrations. Variation in spotting volumes ranging from 20-100 µL resulted in minor biases (range: VEN: −11.4 - 6.4%, ODV: −14.2 - 4.7%). Highest biases were observed for the QC low samples at a spotting volume of either 20 or 100 µL. Linear correlation coefficients between plasma and spiked DBS samples were high (VEN r²: 0.988; ODV r²: 0.984). With Passing and Bablok regression, significant proportional biases were found for both VEN and ODV indicating that concentrations found in DBS samples are higher compared to those from plasma samples. The slopes of the regression lines were 0.83 (CI: 0.79 to 0.93) and 0.76 (CI: 0.72 to 0.83) for VEN and ODV, respectively. The intercepts were not
significant. These results suggest a conversion factor is needed to translate results from DBS to plasma values. This factor is currently investigated as part of the clinical validation procedure.

### 3.2.4. DISCUSSION & CONCLUSION

An novel assay for determination of VEN and ODV in DBS was validated and found to be well within the limits of acceptance. Additionally, DBS specific aspects like influence of Htc, spot volume and punch position were assessed and the relation between plasma and DBS levels was established.
Considerable matrix interference was observed which might indicate positive biased results due to ion enhancement. However, since the calibration curves are prepared as DBS and a low intraindividual CV was found in QC DBS-samples prepared from blood of six different volunteers, no additional correction is needed. Another consideration can be made about the internal standard promazine. Promazine has no marketing authorization on the Dutch market, however it does in some other countries, therefore one might consider using an alternative internal standard. Last, the influence of shipment conditions such as low (−20°C) or high (40°C) temperatures on the DBS stability has not yet been investigated.

**Figure 2.** Effects of Htc on analytical bias (%) of VEN (A) and ODV (B) (n=3 for each point, total n=21).
We assessed Htc effects and found a small negative bias associated with a lower Htc. This negative relationship was also found in other studies, and is thought to be caused by the lower viscosity of blood at a lower Htc [7]. Our results showed that a correction for Htc is needed if Hct < 0.25, but since such low Hct values are very unlikely in the average psychiatric patient population, no correction for Htc seems indicated. Moderate variation in spotting volume did not influence analytical bias. This is beneficial for the use of the method in clinical practice. The same holds for the punch position. Nevertheless, a maximum of 10% bias was observed when punching at the perimeter of the spot and punching at the center of the spot is therefore advised.

A good correlation between the plasma and DBS samples was found with a consistent higher concentration of VEN and ODV in DBS samples compared to plasma samples. Although not reported for VEN or ODV, these differences could be caused by binding of VEN and ODV to red blood cells which is known for other drugs, like for example tricyclic antidepressants (TCA) [9] and confirmed by our finding concerning TCAs as well (results not published yet). Due to the good correlation between our plasma and DBS method (in vitro), a correction factor to convert DBS values to plasma can probably be used, however still needs to be established by clinical validation results.

In conclusion, we analytically validated a new minimal invasive DBS assay, for TDM purposes. Matrix interference was observed, however consistent between the matrixes and the effect could be compensated by using calibration samples prepared as DBS. Effects from Htc, punch position and spot volume revealed acceptable bias. Furthermore, comparison between plasma and DBS samples revealed that higher concentrations of VEN and ODV were measured in DBS samples, which will be further studied as part of the clinical validation of the assay. Currently, this method is used for TDM in the Dutch CYSCE multicenter trial (ClinicalTrials.gov Identifier: NCT01778907) in which the effect of CYP2D6 genotyping combined with TDM on time to reach adequate blood drug levels is investigated in depressed elderly patients.
3.2.5. REFERENCES


