GC-MS identification and UHPLC determination of clozapine in blood samples

Georgi Bonchev, Ivelina Stefanova, Snezha Zlateva, Petko Marinov and Ivaylo Vazharov

Abstract
The main purpose of the work was to optimize a GC-MS screening procedure for identification of low blood clozapine levels and to develop and validate a simple HPLC technique for its determination in biological samples. Gas chromatography in tandem with mass spectrometry, combined with preanalytical purification (mixed-mode solid-phase extraction) and derivatization (PFPA acylation) has been chosen as the method for analytical identification. It has been shown that derivatization procedure is vital component of clozapine and its metabolites identification by GC-MS. Reverse-phase liquid chromatography has been used as a quantitative instrument. HPLC determination has been done under isocratic conditions on Zorbax Extend-C18 column (150x4.6 mm, 5 μm); mobile phase – acetonitrile: phosphate buffer pH 5.2 = 30:70 v/v; flow-rate of 1.0 mL/min and UV detection at 254 nm. The method has been validated in human plasma/serum samples (75% recovery). Limit of quantitation (LOQ) for clozapine in blood samples has been estimated to 25 ng/mL, along with excellent linearity (Pearson’s r = 0.9999 over 25 to 2000 ng mL−1 range) and precision (RSD 2.9%). A clinical case reported demonstrates the importance of clozapine confirmation and level determination, as in cases of combined acute intoxication it is possible to have a major discrepancy between anamnestic data and clinical picture. A possible drug interaction between clozapine, sertraline and mirtazapine, resulting in toxicity escalation due to their concomitant use has been reported.

Keywords: UHPLC, clozapine, sertraline, mirtazapine, concomitant intoxication, analytical toxicology

Introduction
Clozapine (Leponex and Clozaril are frequently used brand names) is orally taken atypical antipsychotic used mostly in treatment of schizophrenia, especially in patients, who do not respond to conventional neuroleptic therapy [1]. Chemically, it is 8-chloro-11-(4'-methyl)piperazino-5H-dibenzo[b,e]-1,4-diazepine; it belongs to dibenzodiazepine class (Fig. 1), and is produced by Novartis International AG.

![Chemical structures of clozapine (I) and its major active metabolite, N-desmethylclozapine (norclozapine, II)](image)

Although being a first atypical antipsychotic [2] and commercially available for more than 40 years now [3] it is still highly praised due to its efficacy; it is believed that clozapine is more effective than typical antipsychotics [4, 5] and is a medicine of choice in treatment-resistant cases [6]. However, therapeutic (300-600 ng mL−1), toxic (600-1000 ng mL−1), and comatose (>1200 ng mL−1) blood levels [7] are very close, elimination half-life is ca. 14 h, and cases of acute clozapine intoxications are not rare [8]. Research on the concomitant intoxications...
(in usual TS scenario) lead to conclusion, that combinations with other psychoactive drugs, and especially benzodiazepines \cite{9}, are potentially fatal. That is why the identification and monitoring of the clozapine in blood is important and should be made as early as possible when over dosage or suicide attempt is registered. Quantitative blood levels are usually determined by high-performance liquid chromatography in tandem with ultra-violet detection \cite{10,11} or by mass-spectrometry \cite{12}.

Materials and methods

A subject of clinical study was male patient (74) of Clinic for Intensive Treatment of Acute Intoxications and Toxicological hospital – Varna, Bulgaria. Human blood and plasma from controlled stationary patients of Naval Hospital – Varna, available in Clinical Laboratory, were taken at random and used for preparation of spiked samples. All of the chemical reagents used were of analytical grade or better. The necessary solutions are prepared using HPLC grade solvents and purified deionized water (0.067-0.100 μS cm\(^{-1}\), TKA™ Pacific water purification system). Pentafluoropropionil anhydride (for GC derivatization, 99%) was purchased from Sigma-Aldrich Co. LLC. Solid-phase extraction was done via Phenomenex \(^{®}\) cartridges (Strata\(^{®}\) Screen-C, 55μm, 70Å, 150 mg/3mL tubes). Certified reference material (Quick-Check™ Drug Solutions of 1 mg mL\(^{-1}\) clozapine in MeOH) was purchased from Alltech Associates, Inc., USA.

GC-MS analysis was done on Agilent Technologies 7890B GC System & 5977A MSD module. Data acquisition and processing were controlled by Agilent MassHunter software package. Reference data from mass spectral library NIST version 2.0 g was used for comparison. UHPLC analysis was done by means of Agilent 1260 Infinity Binary LC featuring Zorbax Extend-C18 column (150 x 4.6 mm, 5 μm) and 1260 Infinity DAD. The Agilent Chem Station package was used for data acquisition and manipulation. Statistical analysis was done using OriginPro\(^{®}\) software.

Results and discussion

Experimental procedure

Qualitative identification of clozapine and its major (active) metabolite – norclozapine was done by means of GC-MS. Temperature program as well as other working parameters are listed in Table 1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial oven temp.</td>
<td>50 °C</td>
<td>GC Column</td>
<td>DB-1701</td>
</tr>
<tr>
<td>Initial time</td>
<td>2 min</td>
<td>Column dimensions</td>
<td>30 m × 0.25 mm</td>
</tr>
<tr>
<td>Oven ramp rate</td>
<td>20 °C min(^{-1})</td>
<td>Film thickness</td>
<td>0.25 μm</td>
</tr>
<tr>
<td>Oven first ramp</td>
<td>90 °C</td>
<td>Inlet mode</td>
<td>splitless</td>
</tr>
<tr>
<td>Final first ramp</td>
<td>1 min</td>
<td>Flow mode</td>
<td>constant flow</td>
</tr>
<tr>
<td>Oven ramp rate</td>
<td>8 °C min(^{-1})</td>
<td>Flow rate</td>
<td>1.5 mL min(^{-1})</td>
</tr>
<tr>
<td>Oven temp.</td>
<td>280 °C</td>
<td>Carrier gas</td>
<td>He</td>
</tr>
<tr>
<td>Final time</td>
<td>15 min</td>
<td>Ion source temp.</td>
<td>230 °C</td>
</tr>
<tr>
<td>Total run time</td>
<td>43.75 min</td>
<td>Inlet temp.</td>
<td>250 °C</td>
</tr>
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In order to enhance the sensitivity and be able to give satisfying results in low-volume low-concentration blood samples, we used a preliminary solid-phase extraction, followed by chemical derivatization by acylation. Detailed description of procedure follows. Condition the SPE column applying 2 mL MeOH followed by 2 mL water. Prepare the sample by diluting 1 mL blood serum/plasma with 2 mL of water. Load the sample at rate as low as 1 drop per second. Wash the column consequently by 2 mL 0.1 M HCl and 2 mL MeOH. Dry at full vacuum for 3 minutes. Elute drop wise using 2 mL 5% ammonium hydroxide in MeOH. Thoroughly evaporate under nitrogen stream (at < 60 °C) to complete dryness. Reconstitute the residue in 50 μL of toluene: acetonitrile mixture (95:5 v/v) and add 50 μL acylation agent (PFPA). Heat for 20 min at 70 °C (keep closed). Evaporate resulting solution under nitrogen stream (at < 60 °C) again. Reconstitute in 30 μL ethyl acetate and inject 1 μL of final solution into the GC column. Pentafluoropropionyl derivatives were identified as follows: clozapine × PFP at \(m/z = 29.81\) min, mass spectrum (EI, 70 eV), \(m/z = 70, 83, 191, 191, 415, 373, 226, 254\) and norclozapine × PFP at \(m/z = 32.96\) min, mass spectrum (EI, 70 eV), \(m/z = 604, 191, 402, 227, 216, 231, 200, 457\).

Quantitative determination was done by HPLC. Pre-analytical procedure begins with 500 μL of blood serum/plasma sample, utilizes alkaline liquid-phase extraction by 2 x 2 mL ethyl acetate, followed by evaporation and reconstitution in 500 μL of mobile phase, followed by syringe filtering (0.22 μm, Nylon). Liquid chromatography was done under isocratic conditions with mobile phase consisting of phosphate buffer (pH 5.2; 10 mM) containing 1.5 mL L\(^{-1}\) triethylamine – acetonitrile (65:35, v/v) at 25°C; flow-rate: 1.0 mL/min. Injected sample volume was 20 μL. Detection was done by UV (diode-array detector, 254 nm). Retention time under described conditions was approx. 5.7 min.

Method validation

Calibration procedure for UHPLC determination was based on the method of external standard. Using a certified material, a set of progressive dilutions was prepared to cover concentrations of toxicological interest, from 25 to 2000 ng mL\(^{-1}\). Excellent linearity (Pearson's \(r = 0.9999\)), precision (RSD of 2.9%, \(n=6\)) and reproducibility (RSD of 3.5% over one week period) were achieved (Fig. 2). LOQ was established at 25 ng mL\(^{-1}\). Although better sensitivity is still within reach, it is out of interest, as lower accepted therapeutic blood level equals 300 ng mL\(^{-1}\). By analyzing spiked blood samples a 75% recovery was determined.
Clinical case

Clinical course of the intoxication: progressive deterioration during the first 2-3 days with cerebral toxic syndrome – depression of the CNS to sopor and coma II degree, cardiotoxic syndrome – supraventricular tachycardia and symptoms of acute left ventricular heart failure, febrile syndrome up to 40 °C, respiratory problems – dyspnea and left side pneumonia. After the treatment these complications were managed and the general state was improved.

Fig 3: Monitoring the blood clozapine levels during the treatment course

Discussion
Although the initial information was about poisoning with clozapine only, the severe clinical deterioration, combined with analytical toxicology report for moderate clozapine blood levels (327 ng mL⁻¹ at hospital admission, Fig. 3), lead us to the conclusion that the case represents a concomitant intoxication with several psychoactive substances. Additional GC-MS screening procedure confirmed the presence of antidepressants – mirtazapine and sertraline. Differential diagnosis: (1) Malignant neuroleptic syndrome; (2) Acute cerebral vascular accident. Clinical outcome: The patient was discharged on the 8th day without intoxication problems.

Conclusion
A reliable and precise method for HPLC determination of clozapine in biological samples has been developed and validated. It has been demonstrated that the method is suitable for analytical toxicology needs, including emergency cases and treatment monitoring for patients with acute intoxications. It has been shown that concomitant intoxications with other psychoactive medications (e.g. antidepressants) can cause an unexpectedly severe clinical picture and toxicology screening is important in diagnosis clarification.

Acknowledgements
The authors would like to thank the team of Analytical toxicology Lab, Military Medical Academy, Sofia, for valuable support and scientific guidance.

References
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