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Research Article

# Development and Validation of an UHPLC-UV method for the Determination of Agomelatine in Human Plasma and Serum Suitable for Routine Clinical Analysis

**Georgios N Rallis[1], Petros Petrikis[2] and Vassiliki A Boumba[1]***

[1]*Laboratory of Forensic Medicine & Toxicology, Faculty of Medicine, Greece*
[2]*Psychiatric Clinic, University Hospital of Ioannina, Greece*

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***Corresponding author**

Vassiliki A Boumba, Laboratory of Forensic Medicine & Toxicology, Greece, Tel: +302651007724; Email: vboumba@uoi.gr



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## Abstract

Agomelatine is a melatonergic antidepressant approved for marketing in the European Union in February 2009; several methods are published for its determination in human specimens so far based mainly on mass spectrometric detection. Aim of this study is the development and validation of the first method based on UV detection of agomelatine after purification from human plasma / serum with a conventional SPE (onto a Bond Elut Certify cartridge) and separation by a UHPLC system (on a Hypersil GOLD analytical column by a mixture of eluents used in gradient mode). The wavelengths of 230 nm and 245 nm were used for the determination of agomelatine and the internal standard (harmine), respectively. The method was validated according to FDA guidelines. Linearity ranges from 50-800 ng/mL, covering therapeutic and supra-therapeutic levels. Extraction recoveries were 91 and 83% for plasma and serum respectively ($R^2$> 0.9946). The intra-day and inter-days precisions ranged from 4.52-7.63 and 5.25-8.01% and, 9.27-10.15 and 9.53-11.05% for plasma and serum, respectively. LODs and LOQs were 15 and 50 ng/mL for both matrices. Overall, the method is specific for agomelatine, selective towards several antipsychotics, other antidepressants and sedative-hypnotics. Validation studies demonstrated that the proposed UHPLC method meets satisfactory validation criteria, is simple, rapid, reliable, reproducible and easily applicable for routine clinical casework.

## Introduction

Agomelatine is a new antidepressant approved for marketing in the European Union in February 2009 [1]. Its main pharmacological and pharmacokinetic properties are the following [1-4]: it has selective agonist action at melatonin receptors (MT1 and MT2 receptors) and selective antagonist action at serotonin 5HT-2C receptors. It does not affect the uptake of serotonin, adrenaline or dopamine. Binding studies indicate that agomelatine has no effect on monoamine uptake and no affinity for adrenergic, histaminergic, cholinergic, dopaminergic and benzodiazepine receptors. Agomelatine is rapidly and well (80%) absorbed after oral administration. Absolute bioavailability is low (<5% at the therapeutic oral dose) and the inter-individual variability is substantial. The bioavailability is increased in women compared to men. The bioavailability is increased by intake of oral contraceptives and reduced by smoking. The peak plasma concentration is reached within 1 to 2 hours. In the therapeutic dose-range, agomelatine systemic exposure increases proportionally with dose. At higher doses, a saturation of the first-pass effect occurs. Food intake (standard meal or high fat meal) does not modify the bioavailability or the absorption rate. The variability is increased with high fat food. Elimination is rapid, the mean plasma half-life is between 1 and 2 hours and the clearance is high (about 1,100 ml/min) and essentially metabolic. Excretion is mainly (80%) urinary and in the form of metabolites, whereas unchanged compound recovery in urine is negligible. Kinetics is not modified after repeated administration.

Up to date limited methods have been reported in the literature for the determination of agomelatine in human specimens [5-9], based mainly on high cost mass spectroscopic techniques [5,6,8,9], while most of them were developed in order to apply to pharmacokinetic studies [5-7,9]. Therefore, the aim of the present study was to develop and fully validate a simple, relatively low-cost UHPLC-DAD method, based on SPE, for the analysis of agomelatine in plasma and serum samples suitable for application in routine clinical casework.

## Materials and Methods

### Chemicals and Reagents

Agomelatine and harmine (internal standard) were purchased from Sigma-Aldrich GmbH

(Steinheim, Germany). Acetonitrile, methanol and water were UHPLC grade and purchased from Fisher Scientific (Loughborough, United Kingdom). Formic acid 99% for analysis was purchased from Carlo Erba (Arese, MI, Italy). Acetic acid (glacial) and ammonia solution 25% were purchased from Merck KGaA (Darmstadt, Germany). Ammonium acetate for UHPLC was purchased from Sigma-Aldrich GmbH (Steinheim, Germany). Other all chemicals were analytical grade. For Solid-Phase Extraction (SPE) Bond Elut Certify (3 mL aqueous capacity, sorbent mass 130 mg) cartridges were used (Agilent Technologies, Lake Forest, CA, USA). Syringe-driven filter units, non-sterile, Millex-GV, 13 mm (PVDF membrane, 0.22 $\mu$m) were purchased from Merck KGaA (Darmstadt, Germany). Autosampler vials were purchased from Thermo Fisher Scientific Inc.

### Standard and Working Solutions

Standard stock agomelatine and internal standard (harmine) solutions at a concentration of 1 mg/ml were prepared by dissolving the appropriate amount of pure analyte in acetonitrile (UHPLC grade). Working solutions of agomelatine in the concentration range 25 ng/mL to 20 $\mu$g/mL (25, 50, 100, 250, 500, 1000, 2500, 5000, 10000, 20000 ng/mL), were prepared by proper dilution of the standard stock solution with acetonitrile and were used for testing the response of the detector. Stock solutions were stored light protected at -20°C. Standard and calibration solutions were prepared at concentrations 50, 100, 200, 400, 600 and 800 ng/mL by spiking 2.0 mL of analyte free human plasma with 80 $\mu$L of the 20 $\mu$g/mL agomelatine working solution and consequent dilutions with plasma.

### UHPLC-UV/DAD Analysis and Instrumentation

Chromatographic analysis was carried out using a UHPLC system Thermo Scientific Dionex Ultimate 3000 equipped with a diode array detector UV/VIS, DAD-3000RS (Dionex Softron GmbH Part of Thermo Fisher Scientific Inc. Dornierstraße, Germany). The system comprised an SRD-3400 solvent rack, an HPG-3200RS binary pump, a WPS-3000TRS autosampler with a steady temperature at 5°C. Analyte was separated with a Hypersil GOLD analytical column (100 mm x 2.1 mm i.d., particle size 1.9 $\mu$m) also supplied by Thermo Fisher Scientific equipped with a precolumn cartridge (2.1 mm X 0.2 $\mu$m). The temperature of the guard and analytical column was maintained at 30°C, while post-column temperature was set at 20°C, with a TCC-3000RS thermostat. Mobile phase was constituted by ammonium acetate 10 mM in 0.1% formic acid (Eluent A) and in acetonitrile (Eluent B). Flow rate was 0.3 mL $min^{-1}$. The gradient program used was as follows: step 1: 15% Eluent B from 0.0 to 1.0 min; step 2:15% to 55% of Eluent B during 8.0 min; step 3: 55% to 100% B from 9.0 to 10.2 min, then washing with 100% B for 0.2 min; and, step 4: a reconstituting step at the initial conditions (15% B) from 10.4 to 13.5 min. Total acquisition time was 10.2 min. The maximum inlet pressure was 4500 psi. The stability of buffer solutions was checked and found to remain stable for at least one month at room temperature. Diode array detector was set to measure the specific wavelengths of 230 nm and 245 nm for agomelatine eluted at 4.77 min and harmine (I.S.) eluted at 7.9 min respectively. The data acquisition and integration was performed by Chromeleon 7.2 Chromatography Data System, Reversion 1.1 (Thermo Fisher Scientific Inc.).

### Sample Handling

For preparation of calibration and validation standards specimens were collected from healthy, drug-free donors. Real samples were obtained from patients at the Psychiatric Clinic of the University Hospital of Ioannina. Plasma samples were retained after centrifugation of human blood collected in Vacutainer tubes with $K_2$EDTA or $K_3$EDTA as anticoagulant at 4,000 rpm for 10 min; serums were obtained after centrifugation of 5 mL blood in BD Vacutainer SST II Advance tubes, (Becton Dickinson) at 2500 rpm for 10 min. Specimen from each individual were divided into aliquots of 1.0 mL, frozen immediately and stored at -20°C until analysis. Samples could be kept frozen up to six months.

### Sample Preparation

Calibration standards as well as real samples were extracted by Solid-Phase Extraction (SPE). The extraction conditions were set as reported below: 1.0 mL of biological fluid in conical glass tubes, were fortified with 100 ng of harmine (IS) (20 $\mu$Lof 5 $\mu$g/mL). The mixture was diluted with 2.0 mL of 0.2 M phosphate buffer ($NaH_2PO_4$), pH 6.0. Then the tubes were vortex-stirred, left to stand for 5 min at 4°C and centrifuged at 4,000 rpm for 15 min. The supernatant was collected and loaded onto the SPE Bond Elut Certify cartridge, pre-conditioned with 3.0 mL methanol, 3.0 mL Double Distilled (DD) water and 1.0 mL of 0.1M potassium diphosphate adjusted to pH 6.0. The loaded SPE column was washed with 3.0 mL of DD water acidified with 1.0 mL of 1.0 M acetic acid and finally was dried under vacuum. Elution of analytes was performed adding twice, 2X1.5 mL of a mixture of methanol: 25% ammonia solution (98:2, v/v) by gravity flow. The eluent was evaporated to dryness under a gentle stream of nitrogen at 40°C, reconstituted in 100 $\mu$L of mobile phase, Eluent A : Eluent B (50:50, v/v), filtered and transferred to an autosampler vial. Finally, 5.0 $\mu$L from the final extract was injected into the UHPLC-UV/DAD system.

### Method Validation

The analytical method was validated for human plasma and serum in accordance with the international guidelines [10]. The method was tested for selectivity, specificity and stability and validated for linearity, accuracy, precision, limits of detections and quantification.

Stability was tested in solutions of agomelatine in acetonitrile by comparing the chromatographic peak areas of a standard solution of 100 ng/mL from the stock solutions stored for 2 months at -20°C, with those obtained from the fresh stock solutions (n = 3). Stability assays were also carried out in human serum and plasma samples spiked with agomelatine at 100 ng/mL. The peak areas of extracted plasma and serum samples, stored for up to 72 hours at 4°C, were compared with those of aliquots of the same samples when freshly extracted and chromatographed (n = 3). Also, the peak areas of extracted plasma and serum samples, after three freeze-thaw cycles and after storage for 6 months at -20°C, were compared with the peak areas of extracts of aliquots of the same samples when fresh (n = 3).

## Results

The developed method is suitable for the specific and sensitive determination of agomelatine in human plasma and/or serum by UHPLC-DAD after SPE. Harmine was used as internal standard because it is a Central Nervous System (CNS) acting compound having similar chemical properties to agomelatine and it is neither a prescribed nor an abused drug (Figure 1). Chromatographic

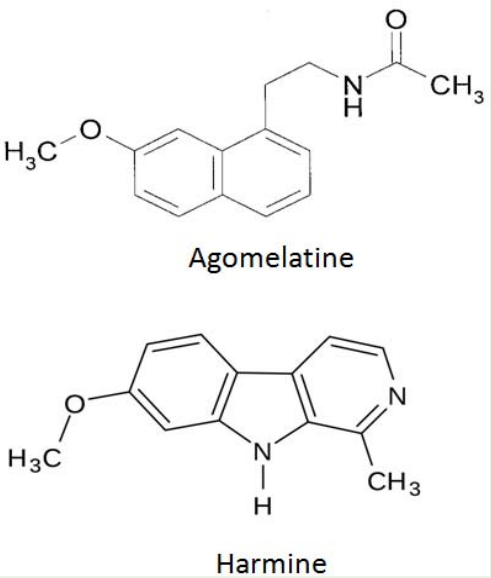


**Figure 1:** Chemical structures of: (A) agomelatine and, (B) harmine (internal standard).

conditions, such as mobile phase, gradient conditions, column temperature, and extraction conditions were optimized. Therefore different mixtures of organic solvents (acetonitrile, methanol, water) in various proportions were tested as eluents in order to achieve sufficient extraction, clean extracts and minimum matrix effect. The choice of SPE was based on previously published reports on the extraction of antidepressants from plasma samples [11-13].

Selectivity towards xenobiotics was evaluated by injecting into the UHPLC system standard solutions of several compounds active on the CNS, such as antipsychotics (e.g. olanzapine, quetiapine, risperidone, paliperidone, amisulpride, haloperidol, clozapine, norclozapine), other antidepressants (e.g. mirtazapine, citalopram, desmethyl-citalopram, clomipramine, sertraline, bupropion, venlafaxine, fluoxetine, paroxetine) and sedative-hypnotics (e.g. diazepam, bromazepam, nordiazepam, oxazepam). None of the tested compounds interfered with the chromatographic peak of agomelatine or harmine within the chromatography time of 10.2 min, in the concentration level of 1000 ng/ mL, using the respective retention time as criterion of specificity. To assess endogenous compound selectivity, blank plasma/serum samples, obtained from six different healthy volunteers of different ages, with and without internal standard, were subjected to the sample preparation and analysis procedure. None of these samples showed any peak, which interfered with the analysis of agomelatine or harmine.

The optimized method was evaluated by characterizing its analytical performance in terms of linearity, precision, and recovery, Limit of Detection (LOD) and Limit of Quantification (LOQ). The results are listed in Table 1. The calibration curves were obtained in human plasma and serum, fortified with agomelatine at six concentration levels (50, 100, 200, 400, 600 and 800 ng/mL), by plotting peak area of agomelatine versus the corresponding agomelatine concentration in the specimen. The calibration curves were constructed by means of the least square method. The calibration curves were linear in the concentration range of 50-800 ng/mL for both specimens with the coefficients of determination ($R^2$) greater than 0.9946 (Figure 2).

Three replicate standards per concentration level were prepared and processed. Three measurements of each extract were performed on the UHPLC System. The linearity range covers sub-therapeutic, therapeutic and supra-therapeutic (toxic) levels, although toxic concentrations are not definitely specified yet.

The described sample preparation procedure for blank plasma sample spiked with 50, 200, and 800 ng/mL of agomelatine was repeated six times in the same day to obtain intra-day precision. Also, it was analyzed in seven different days (five repeated determinations in each day) to obtain inter-day precision. Both precisions, expressed as percentage relative standard deviations (RSD%), were calculated using response signal values and collected in Table 1. The intra-day precisions were in the ranges of 4.52-7.63% and 5.25-8.01% as well as inter-days precisions for them were 9.27-10.15% and 9.53–11.05% for plasma and serum, respectively. LOD and LOQ were calculated as the concentrations of the analytes giving peaks in which signal-to-noise ratio was 3 and 10, respectively. Both, LOD and LOQ, were determined by analysing six spiked plasma /sera prepared at their respective concentrations. For both specimens the resultant LOD and LOQ were 15 and 50 ng/mL, respectively. The extraction recoveries of agomelatine from plasma and serum, defined as percentage of the extracted amounts of the analyte into the final phase, were 91 and 83%, respectively. The results of the stability experiments (Table 1)

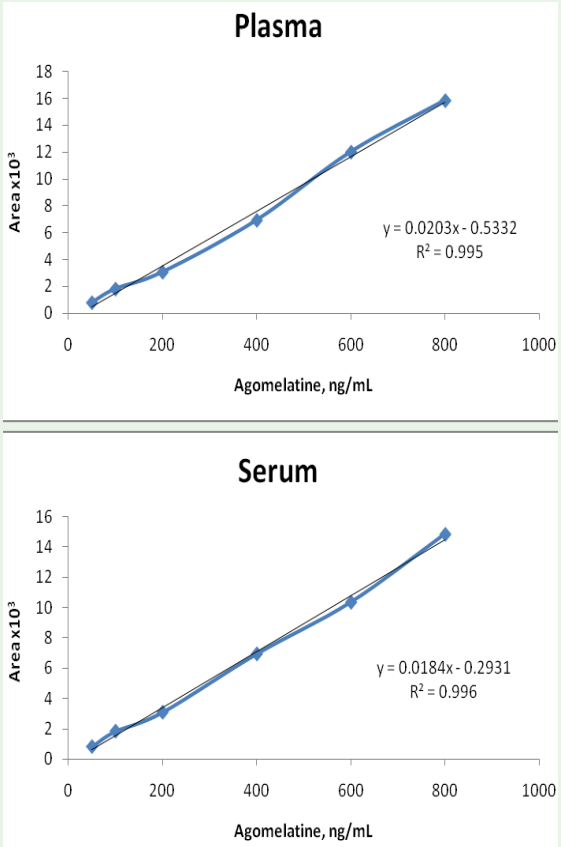


**Figure 2:** Calibration plots and equations for the determination of agomelatine concentration in plasma and serum respectively.

**Table 1:** Validation results for linearity, sensitivity, recovery, stability and selectivity of the current assay.

| Validation Parameter | Plasma | Serum |
|---|---|---|
| **Linearity Range (ng/ml)** | 50 - 800 | |
| **Linearity ($R^2$)** | 0.9946 | 0.9963 |
| **Extraction Recovery % (mean ± SD)** | 91 ± 3.0 | 83 ± 2.2 |
| **Extract stability (at 4°C)** | - 0.4±0.02 | - 0.5±0.04 |
| **Freeze-thaw stability** | -1.1 ± 0.08 | -1.4 ± 0.06 |
| **Long-term stability** | -1.7 ± 0.28 | -1.8 ± 0.17 |
| **Stock solution stability** | 0.3 ± 0.06 | |
| **Selectivity** | *APs**: Olanzapine, Quetiapine, Risperidone, Paliperidone, Amisulpride, Haloperidol, Clozapine, Norclozapine<br>*ADs**: Mirtazapine, Citalopram,Desmethyl-Citalopram, Clomipramine, Sertraline, Bupropion, Venlafaxine, Fluoxetine, Paroxetine<br>*BZDs**: Diazepam, Bromazepam, Nordiazepam, Oxazepam | |
| **LOD (ng/ml)** | 15.00 | |
| **LOQ (ng/mL)** | 50.00 | |

* Abbreviations: APs for antipsychotic drugs; ADs for antidepressant drugs and BZDs for benzodiazepines (sedative hypnotics).

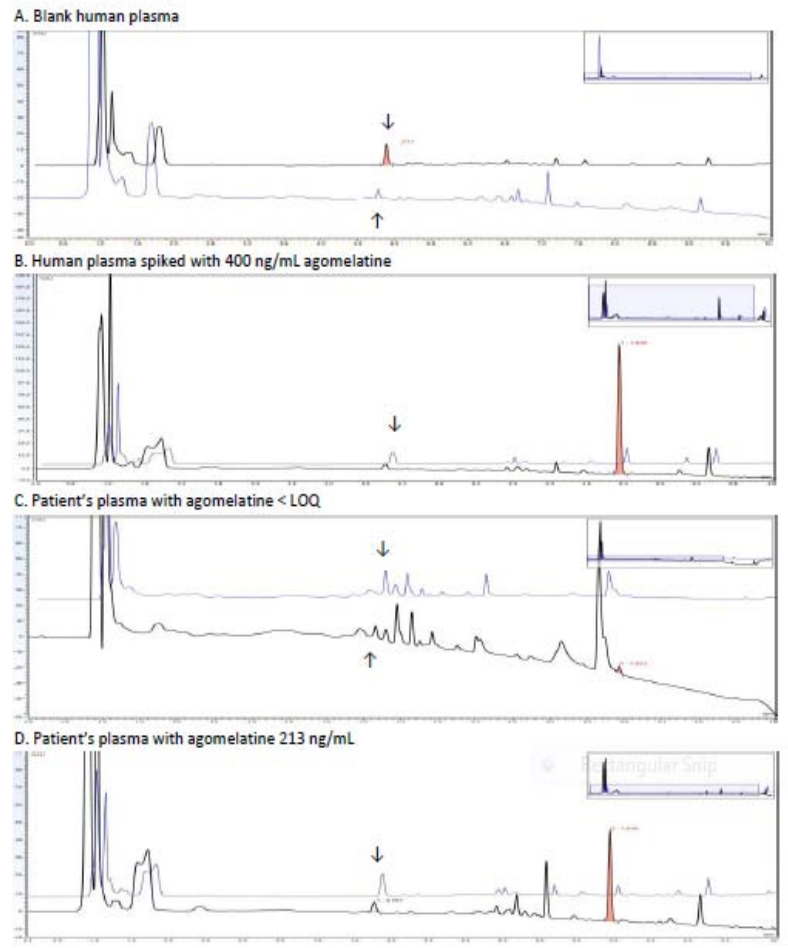


**Figure 3:** Representative chromatograms of: (A) blank human plasma; (B) human plasma spiked with 400 ng/mL agomelatine; (C) patient's plasma with agomelatine concentration lower than LOQ; and (D) patient's plasma with agomelatine concentration 213 ng/mL. All plasma samples were spiked with 100 ng/mL harmine (I.S.) and were processed as described. Lower and upper plots were acquired at 230 nm and 245 nm, respectively. Shaded peaks show: agomelatine in (B), (C) and (D) and harmine (A) at the respective wavelengths used for the determination of each compound. The arrows show harmine. (The inserts show the total chromatograms without magnification of scale).

**Table 2:** Validation results on plasma or serum samples spiked at three concentration levels (50, 200 and 800 ng/mL) performing five determinations per concentration level.

| Validation Parameter | Plasma | Serum |
|---|---|---|
| Linearity Range | 50 - 800 | 50 - 800 |
| LOQ (ng/mL) | 50 | 50 |
| Accuracy (R %), mean ± SD<br>50 ng/mL<br>300 ng/mL<br>800 ng/mL | <br>91.42<br>83.24<br>77.41 | <br>89.55<br>92.74<br>81.36 |
| Extraction Recovery (% ± SD) | 91 ± 3 | 83 ± 2 |
| Intra-day precision (RSD%)<br>50 ng/mL<br>200 ng/mL<br>800 ng/mL | <br>7.63<br>6.86<br>4.52 | <br>8.01<br>5.49<br>5.25 |
| Inter-day precision (RSD%)<br>50 ng/mL<br>200 ng/mL<br>800 ng/mL | <br>9.27<br>10.15<br>9.98 | <br>11.05<br>9.65<br>9.53 |

showed that agomelatine was stable under the tested experimental conditions of storage: (a) in acetonitrile when stock solutions were stored at -20$^0$C for at least 2 months; (b) when the serum and plasma extracts were stored for up to 72 hours at 4$^0$C; (c) when plasma and serum samples up to three freeze–thaw cycles and (d) when stored at –20$^0$C for 6 months.

The accuracy of the suggested method was checked by its performance on plasma or serum samples spiked at three concentration levels (50, 200 and 800 ng/mL) performing five determinations per concentration level. The results are shown in Table 2. These values indicate the presented method's ability in accurate and precise determination of the agomelatine in complicated matrices such as human plasma and serum after pre-treatment according to the stated protocol.

The developed method was applied for agomelatine determination in plasma samples received from psychiatric patients after oral administration of agomelatine (Figure 3). The patients were under agomelatine medication received either alone or in combination with other psychiatric drugs (manuscript in preparation). Figure 3C and 3D show the UHPLC chromatograms of plasma samples containing sub-therapeutic (<LOQ) levels of the drug (sample was received from a patient 12 hours after the last dose of 50 mg agomelatine) and therapeutic levels (213 ng/mL) of agomelatine (sample was taken one hour after dosing 50 mg agomelatine).

## Discussion

A simple, rapid, sensitive and specific method has been developed and validated for the determination of agomelatine in human plasma or serum using UHPLC-DAD instrumentation. The presented method provides innovative characteristics compared to other previously published methods: it is the first study based on conventional SPE extraction for the isolation of agomelatine from plasma /serum, which provides clean extracts and high pre-concentration; and furthermore the detection of analytes is performed with the universal DAD. The developed method was fully validated for its selectivity, specificity, accuracy and precision according to international criteria [10].

Up to date, to the best of our knowledge, four methods have been published for the determination of agomelatine in human plasma [5,6,8,9]. Two of the reported bioanalytical methods [5,6] employed a liquid–liquid extraction procedure and a time-costing evaporation process, and the linear ranges were relatively narrow, which are not adequate for the determination of this high variability drug. The method of Li and colleagues that provides the detection of agomelatine and its metabolites in human plasma although has a simple sample preparation procedure it is based on the expensive LC-MS/MS instrumentation [9]. The high cost equipment is the main disadvantage of the UPLC-MS/MS method reported by Ogawa and colleagues [8] and the liquid chromatography-fluorimetric detection (HPLC-F) assay reported by Saracino and colleagues [7].

The current method uses relatively low cost and widely available in most laboratories instrumentation which combines the selectivity and speed of an UHPLC separation with the simplicity of DAD. The extraction efficiency of the applied SPE procedure is comparable to previously published methods using human plasma or serum specimens. It has a wide concentration linearity range covering mainly therapeutic and supra-therapeutic levels. The proposed method is sensitive and easily applicable in routine clinical casework, when adjustment of the dose of common antipsychotics and agomelatine is required.

## Conclusion

In this article, the feasibility of the use of a conventional SPE in combination with UHPLC separation and DAD as a simple, inexpensive, and reliable method in the determination of agomelatine from human plasma and serum has been examined. Sample preparation includes SPE using Bond Elut Certify columns. Evaluation of the analytical features of the method provided satisfactory linearity, precision, detection limit, accuracy and extraction recovery. The presented method demonstrated many practical advantages such as simplicity, and low cost. The method was successfully applied to plasma sample analysis for routine clinical cases.

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