Development and Validation of a High-performance Liquid Chromatography Method with Ultraviolet Detection for the Determination of Flunitrazepam in Human Plasma

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A high performance liquid chromatography (HPLC) method was developed and validated for determination of flunitrazepam in human plasma. After a simple liquid-liquid extraction, the analyses were carried out on a ODS column with diode array detection at 330nm. The mobile phase consisted in a mixture of potassium dihydrogen phosphate/acetonitrile (40/60, v/v). The method showed good linearity, accuracy and precision. Advantages of this validated assay include a simple plasma extraction method, short analysis time and good sensitivity (LLOQ = 5ng/mL). The stability data indicated a potential instability of flunitrazepam in plasma at room temperature.

Keywords: flunitrazepam, chromatography, validation, stability

Flunitrazepam (5-(-2-Fluorophenyl)-1-methyl-7-nitro-1,3-dihydro-2H-1,4-benzodiazepin-2-one, Rohypnol) is a short acting benzodiazepine 10 times more potent than diazepam, producing sedative-hypnotic effects, muscle relaxation and amnesia. It is one of the drugs most commonly implicated in drug facilitated rapes. Effects of the drug are more concerning when used in combination with alcohol and can lead to anterograde amnesia. Flunitrazepam is used in combination with alcohol and marijuana to enhance their effects. It is the most popular benzodiazepine in the group of heroin addicts and is frequently used to reduce some of the unpleasant symptoms that follow the stimulants’ induced “high” or caused by the withdrawal [1-3].

The analysis of flunitrazepam in biological samples is recommended for the identification of cases of abusive use and of drug-facilitated sexual assaults. The samples recommended to be used are whole blood, plasma, urine, hair and also saliva. The methods described in the literature are as follows:

· HPLC-UV with the disadvantages of poor sensitivity (LLOQ ≥ 10ng/mL) and/or expensive, laborious sample preparation [4-8]
· HPLC-MS/MS with increased sensitivity, but involving high costs and being inaccessible for some laboratories [9-13]. Recently a HPLC/MS/MS method was elaborated for quantification of flunitrazepam and 7-amino-flunitrazepam in human urine after an on-line solid phase extraction, but despite the automatic sample treatment the total run time was very long (22 min/sample) [12].
· GC-MS/MS with increased sensitivity, but with the disadvantages of laborious sample treatment especially because it usually involves a supplementary derivatization step and that is not accessible for all laboratories [4, 14-16].

The stability of the drug in plasma samples is very important especially in forensic cases, because samples are frequently analysed several days after sampling. Other important aspects are those related to the long term stability and freeze-thaw stability of flunitrazepam in human plasma, because in forensic cases it could be necessary to reanalyse the samples for confirmation or counter-valuation.

The stability of flunitrazepam in whole blood has been reported by El Mahjoub and Staub. [17] They have reported the complete degradation of the analyte in case of storage at room temperature for 6 months, and also a significant decrease in flunitrazepam content at -20°C (a decrease of 5-20%).

The aim of this study was:
- to elaborate and validate a rapid and sensitive HPLC method with diode array detection (DAD) for the quantification of flunitrazepam in human plasma
- to apply this method to evaluate the stability of flunitrazepam in human plasma, in order to establish the optimal sample treatment and storage conditions that will guarantee the avoidance of analyte loss.

Materials and methods
Chemicals
All reagents and chemicals were of analytical grade and were from Merck (Darmstadt, Germany) unless stated otherwise. Solvents were of HPLC grade from Sigma-Aldrich (Steinheim, Germany). Flunitrazepam, nitrazepam, diazepam and chlordiazepoxide pure standards were purchased from Sigma-Aldrich. Deionised water was obtained using a Milli-Q water purification system (Millipore, Milford, MA, USA). The human blank plasma was supplied by the Local Bleeding Centre Cluj-Napoca, Romania.

Standard solutions
Primary stock standard solutions were prepared by dissolving each benzodiazepine in methanol to obtain a concentration of 1mg/mL. They were stored at -14°C. Working standard solutions were prepared daily by dilution of stock solutions with deionised water.

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Calibration standards (ranging from 10 to 260ng/mL) and quality control (QC) samples were prepared by addition of the determined quantity of working stock solution to drug free plasma. Plasma QC sample concentrations were of 10.4, 62.5 and 260ng/mL.

Instrumentation and chromatographic conditions

The analysis was performed using a 2695 Waters Alliance HPLC (Waters, Milford, MA, USA) system composed of a ternary pump, autosampler, column heater and solvent degasser. The HPLC unit was linked to a 996 PDA (photodiode array) detector (Waters, Milford, MA, USA).

Chromatographic separation was achieved on a Supelcosil (Supelco, Bellefonte, PA, USA) ODS column (150mm . 4.6mm, 3µm guard column, maintained at 25°C. The mobile phase consisted in a mixture of potassium dihydrogen phosphate (12.7mM, pH = 2.25)/acetonitrile (40/60, v/v). The flow rate was 0.5mL/min and the absorbance of the eluent was monitored at 330nm. After each set of three samples a washing step with gradient elution was needed to eliminate strongly retained endogenous compounds. This gradient included the passage from 60% to 100% acetonitrile in 5 min; it was maintained at 100% acetonitrile for 10min, back to 60% acetonitrile in 5min and equilibrated for 2 min. The sample compartment was maintained at 4°C. The Empower software was used for instrument control, data acquisition and data handling.

Sample preparation

1mL of plasma was extracted with 3.5mL ethyl acetate, after addition of 100µL of the internal standard (I.S.) solution (2µg/mL nitrazepam in Milli-Q water). Samples were vortexed thoroughly for 2 min and centrifuged for 10 min at 1400g (Sigma 2-16 centrifuge, Sigma Laborzentrifugen GmbH, Germany). The organic layer was transferred in a glass centrifuge tube and evaporated using a 5301 Eppendorf concentrator (Eppendorf AG, Hamburg, Germany) at 45°C under vacuum.

The residue was redisolved in 150µL mobile phase (see section 2.3), transferred to an autosampler vial and a 50µL aliquot was injected for liquid chromatographic separation with DAD detection.

Validation of the analytical method

The method was validated according to the FDA Guidance for Bioanalytical Method Validation [18].

To examine the possible interferences of endogenous compounds, six plasma samples from different sources were extracted and analyzed during method validation. These samples were pre-treated according to the sample preparation procedure except for the addition of the I.S.

The linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the concentration of the analyte in the sample. The linearity was evaluated by injecting a total of 7 calibration standards and the calibration curves were constructed by plotting peak area ratios of flunitrazepam and I.S. against corresponding concentrations.

Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. A number of five replicates per concentration were used, corresponding to the concentration levels of the calibration standards. Accuracy was calculated as the percentage difference between the concentration of drug measured from calibration curve and the concentration of drug added to the blank plasma.

Intra- and inter-day precision, defined as coefficient of variation (CV%), was determined using QC samples corresponding to low, medium and high concentrations (10.4, 62.5 and 260ng/mL). Five replicates were analyzed at each concentration on three consecutive days.

The recoveries of flunitrazepam and I.S. from plasma were determined by comparing the analytical results for extracted samples at all seven calibration levels with unextracted standards that represents 100% recovery.

The lower limit of quantification (LLOQ) was defined as the lowest concentration with a precision of less than 20% CV and an accuracy of 80 to 120%.

Stability

To ensure the reliability of the results in relation to handling and storing of plasma samples and stock standard solutions, stability studies were carried out at two different concentration levels (10.4ng/mL and 260ng/mL flunitrazepam).

The long-term stability of flunitrazepam in plasma was assessed after 1, 3 and 6 months of storage at -14°C. The short-term room temperature stability was examined by keeping the replicates of the low and high concentration plasma samples at room temperature, protected from light and by analyzing the QCs after 24, 48 and 72h. Freeze-thaw stability was determined during 3 cycles, by thawing QCs at room temperature for 1h and then refreezing at -14°C for 24h. The stability of final extracts was tested at 4°C in the autosampler after 24h.

For all storage conditions plasma samples were analyzed in triplicate for each concentration and at each time point.

The stability of flunitrazepam and I.S. stock solutions was determined at -14°C for 41 days and 33 days, respectively.

Results and discussion

Chromatography

Using the HPLC conditions described, flunitrazepam and nitrazepam (fig.1) are well resolved and elute in symmetrical peaks. The retention times of flunitrazepam and I.S. are 6.2min and 5.4min, respectively.

For the selection of I.S. other benzodiazepines were injected separately (nitrazepam, diazepam and chlordiazepoxide). Based on the retention times of these compounds and on the interferences from endogenous compounds, nitrazepam proved to be the best choice for I.S. figure 2 shows the chromatogram of a mixture of flunitrazepam, nitrazepam, diazepam and chlordiazepoxide.

The HPLC-UV methods described in the literature are based on the measurement of absorbance at 220nm, 240nm, 242nm or 250nm [4-8]. At these wavelengths we observed a significant interference from the endogenous compounds. The possibility of using a diode array detector allowed us to select the appropriate wavelength at 330nm, with increased selectivity and signal to noise ratio (sensitivity).
For the determination of flunitrazepam and I.S., a simple, inexpensive and robust single-step extraction procedure was developed. The obtained extraction recovery results were around 60% and they were good enough to guarantee a great sensitivity.

During the optimization of the extraction procedure three solvents were tested - ethyl acetate, 1-chlorobutane and diethyl ether - with or without adjusting the pH with 25% NH₄OH. The best results with good recovery and insignificant interferences were obtained with ethyl acetate without adding NH₄OH.

Method validation

The assay was found to be selective for flunitrazepam and I.S. as no interfering peaks were observed in the extracts of the different blank plasma samples (fig. 3). The other benzodiazepines tested as potential I.S. were found not to interfere with both flunitrazepam and nitrazepam retention times.

Calibration curves were linear in the concentration range investigated with a slope (reported as mean ± SD) of 0.5467 ± 0.0043, a correlation coefficient of 0.9991 ± 0.0007, and y-intercept of -0.0047 ± 0.0034.

The accuracies for flunitrazepam were found to be within 90.9% and 113.0%, while the biases ranged from -6.8% to 8.6%. The recovery of flunitrazepam and I.S. in plasma was 59.3% and 57.0%, respectively. The intra- and inter-day precision (CV %) for flunitrazepam in plasma (table I) were d ≤ 6.1%.

The lower limit of quantification (LLOQ) of the method was determined empirically by running a series of QC samples.
plasma samples containing flunitrazepam over the concentration range of 1-10ng/mL (fig. 4). The LLOQ for flunitrazepam was found to be 5ng/mL (Accuracy = 116.1%, CV % = 13.06%).

Stability data
The aim of the stability studies was to evaluate all possible exposures that the samples might experience after collection until the results of the analysis are obtained.

The primary stock solution of flunitrazepam was stable at least for 41 days when stored at -14°C (concentration of the analyte was found to be 95.4% of the initial concentration). The primary I.S. stock solution was found to be stable for at least 33 days in case of the same storing conditions (the concentration found was 108.7% of the initial value).

Plasma sample extracts maintained at 4°C in the autosampler for 24h, indicated no significant change in the concentration levels of the respective analyte (the mean changes in concentrations were of -2.42 and -0.92% for the 10.4ng/mL and 260ng/mL QC samples, respectively).

The results obtained after 72h in case of short-term stability study of plasma samples at room temperature protected from light demonstrated the recovery of 86.77 and 94.40% of the initial content of analyte (for the low and high concentration, respectively), indicating a potential instability under these conditions.

The residual percentages of flunitrazepam in plasma stored at -14°C for 6 months ranged from 95.18 to 101.27% of the initial concentration, indicating that no long-term stability problems occurred in the mentioned storage conditions.

The mean changes in flunitrazepam concentration after three freeze-thaw cycles were of -4.45 and 7.49% for the 10.4ng/mL and 260ng/mL samples, respectively.

Conclusions
A novel HPLC method with ultraviolet detection for the determination of flunitrazepam in human plasma was developed in this study. The method is rapid, simple and suitable for routine analysis. The advantages of the assay are short time of analysis, a simple extraction and a LLOQ (5ng/mL) lower than that reported in the literature for other HPLC-UV methods [5-8]. The method was validated over a concentration range of 10-260ng/mL and it offers good accuracy and precision for monitoring the cases of abusive use of flunitrazepam. The range of linearity makes this method suitable for forensic and clinical purposes. Although lower LLOQ have been reported when using methods such as liquid chromatography or gas chromatography coupled with mass spectrometry, and probably these techniques are of choice for the confirmation of drug abuse, other techniques may be also acceptable, if selectivity and adequate sensitivity, accuracy and precision are demonstrated. In this context, the HPLC with UV (DAD) detection is a useful technique, mainly because of the lack of a derivatization step (when compared to GC methods) and its lower costs, advantages that are noteworthy in developing countries.

Regarding the stability of flunitrazepam in plasma, a potential instability could be noted when plasma samples are stored at room temperature. It is recommended to analyze the samples as soon as possible after collection, otherwise they must be stored at temperatures under -14°C to guarantee the stability of the analyte, since the metabolization of flunitrazepam even after the sample has been drawn from the body was reported earlier in the literature [19].

References

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