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Short communication

# Validated method for the therapeutic drug monitoring of flunitrazepam in human serum using liquid chromatography–atmospheric pressure chemical ionization tandem mass spectrometry with an ion trap detector

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**Abstract**

An HPLC–MS–MS method for the quantitative analysis of flunitrazepam in human serum was established. The method features a very simple liquid–liquid extraction, the use of a standard 4-mm HPLC column, isocratic elution using a buffer-free solvent, short retention times in connection with good peak resolution and the sensitivity and selectivity of an ion trap MS–MS detector. The procedure enables unambiguous identification of analytes by their product ion spectra, as well as sensitive quantitation (limit of quantitation for flunitrazepam=0.5 ng/ml). This feature was used for the confirmation of HPLC–UV results for nitrazepam. 

*Keywords:* Flunitrazepam

## 1. Introduction

Flunitrazepam (FNZ), being one of the most potent hypnotic benzodiazepines, has clinical importance for the induction of general anesthesia. On the other hand, it has often been misused as an incapacitating agent in rape or robbery [1]. Due its high sedative potency, the effective concentrations in serum are rather low (1–15 ng/ml) [2]. A sensitive and selective method for the determination of FNZ in human serum or plasma is therefore necessary for unambiguous identification and quantitation. The importance of therapeutic drug monitoring is founded on a clear correlation between serum concentration and clinical effects [3]. A good correlation has been demonstrated between amnesia and plasma concentration of FNZ. And a low concentration has been related to postanaesthetic nausea [4]. Whereas HPLC with UV detection is not sensitive and specific enough [limit of detection (LOD)=10 ng/ml [5]], GC–MS suffers from the thermal instability [6] and low volatility of many benzodiazepines, requiring derivatization steps. The coupling of HPLC and MS is a promising technique for fast, sensitive and specific analysis of FNZ and related benzodiazepines. Several methods for the determination of FNZ using LC–MS (or LC–MS–MS) with either electrospray ionization (ESI) [7] or atmospheric pressure

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chemical ionization (APCI) were published recently [8,9]. Kleinschnitz et al. [7] used their ESI–MS–MS method for the determination of several benzodiazepines with a triple–quadrupole instrument. The APCI method of Bogusz et al. [8] was optimized for FNZ and several of its metabolites using a single–quadrupole instrument with so-called skimmer-collision induced dissociation (CID). Although analyte fragments can be detected, this is, with regard to selectivity, is not an equivalent to the second mass spectrometric stage of a triple–quadrupole instrument because fragments are formed from any substance eluting from the HPLC. On the other hand, Bogusz et al. [8] stated that the APCI interface leads to a seven times higher sensitivity when compared to an ESI method. Thus, a combination of MS–MS with APCI should give optimal results.

A solid-phase extraction (SPE) is applied in both references as the sample preparation. A liquid–liquid extraction would be a less expensive alternative.

Based on a validated HPLC–UV method for nitrazepam (NZP) and clonazepam (CLN) used in our laboratory, we developed a method for the determination of FNZ using liquid–liquid extraction as the sample preparation and an ion trap mass spectrometer as the LC detector. The detector would give MS–MS opportunities at an affordable price compared to triple stage quadrupole MS systems. The suitability of an ion trap has to be proven because this was doubted at least in the GC–MS–MS analysis of benzodiazepines [10]. The APCI method reported here was validated and applied within routine therapeutic drug monitoring. Results of a patient sample are presented. The ability of the detection technique to serve as a confirming method for the HPLC–UV determination of NZP and CLN is discussed.

## 2. Experimental

### 2.1. Reagents

FNZ and CLN were supplied by Lipomed (Arlesheim, Switzerland), NZP was a kind gift from Arzneimittelwerk Dresden (Radebeul, Germany). Acetonitrile (ultra gradient grade) was supplied by J.T. Baker (Gross-Gerau, Germany). KCl, $H_3BO_4$, NaOH, *tert.*-butyl methyl ether and methanol were obtained from Merck (Darmstadt, Germany) at analytical-grade. Distilled tap water was purified by a Barnstaedt EasyPure system (Werner, Leverkusen, Germany). A borate buffer (pH 9) was prepared by mixing 1 l of a solution containing 6.2 g of $H_3BO_4$ and 7.5 g of KCl in water with 420 ml of 0.1 *M* NaOH.

### 2.2. Stock solutions and calibration samples

Stock solutions for each benzodiazepine were prepared by dissolving 10 mg of the analyte in 100 ml of methanol. For the determination of FNZ, these stock solutions were further diluted with water to give concentrations of 0.4 and 0.2 μg/ml for FNZ and CLN (internal standard, I.S.), respectively. A 4.9-ml aliquot of water was added to 100 μl of the respective stock solution for the determination of NZP or CLN. In these cases FNZ served as the I.S. Its stock solution was diluted to give a concentration of 400 μg/ml.

Calibration samples were prepared by adding known amounts of the analyte to drug-free human serum. The calibration ranges were 0.8–16 ng/ml for FNZ and 10–100 ng/ml for NZP. Calibration curves were constructed by linear regression using the peak area ratios of analyte to internal standard.

### 2.3. Sample preparation

For extracting a sample, 1 ml of borate buffer (pH 9), 50 μl of the respective I.S. solution and 3 ml of *tert.*-butylmethyl ether were added to 1 ml of the serum. The sample was extracted for 10 min on a roller mixer and centrifuged for 10 min at 1000 *g*. The supernatant organic phase was transferred to a conical glass tube and evaporated to dryness under vacuum. The residue was redissolved in 100 μl of an acetonitrile–water mixture (35:65, v/v) and transferred to autosampler vials fitted with 250-μl inserts.

### 2.4. Instrumentation

#### 2.4.1. HPLC

For HPLC, a Hewlett-Packard HP1100 system consisting of a degasser, a binary pump, an autosampler, a column oven and a diode array detector (Hewlett-Packard, Waldbronn, Germany) was used.

The separation was performed on an RP-18 end-

capped Superspher 100 125×4 mm (Merck, Darmstadt, Germany) column with a guard column and acetonitrile–water (40:60, v/v) as the HPLC solvent at flow-rate of 1 ml/min.

*2.4.2. Mass spectrometry*

The mass spectrometer used was an LCQ ion trap detector equipped with an APCI interface (ThermoQuest, Egelsbach, Germany). The temperatures of the vaporizer and the heated transfer capillary were set to 500°C and 150°C, respectively. The source current was set to 5 μA. All other source parameters (capillary voltage, gas flow, collision energy, etc.) were optimized by constantly adding FNZ to the HPLC flow by a syringe pump via a T-connector.

In the MS–MS experiments the protonated molecular ion $[M+H]^+$ was fragmented. All investigated benzodiazepines gave an intensive product ion at $[M+H\text{-}46]^+$ corresponding to loss of their nitro group.

## 3. Results

Product ion chromatograms of certain calibration samples are presented in Fig. 1. As expected from the high selectivity of the MS–MS technique, no interference with the analytes was observed. The signal response was linear over the investigated concentration range (0.2–20 ng/ml) covering the therapeutic concentrations of FNZ in humans [2]. A five-point calibration from 0.8 to 16 ng/ml was applied for routine therapeutic drug monitoring of FNZ in samples of patients (see Fig. 2 for an example). The LOD was found to be 0.19 ng/ml based on the confidence and prognosis intervals obtained from a ten-point calibration in the range of 0.2–2 ng/ml. The signal-to-noise ratio (SNR) at 0.2

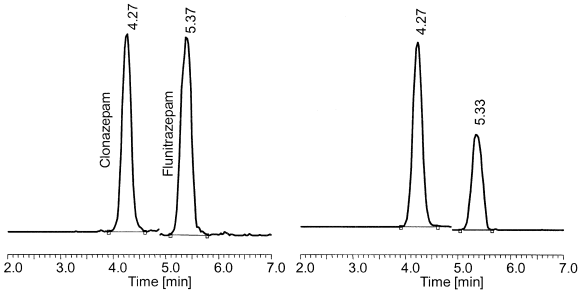


Fig. 2. Product ion chromatograms of a patient sample (left, calculated concentration=1.7 ng/ml FNZ) and a calibration sample (right, spiked concentration=0.8 ng/ml FNZ). The monitored product ions were *m/z* 270 for clonazepam and *m/z* 268 for flunitrazepam. Both chromatograms are shown on the same scale.

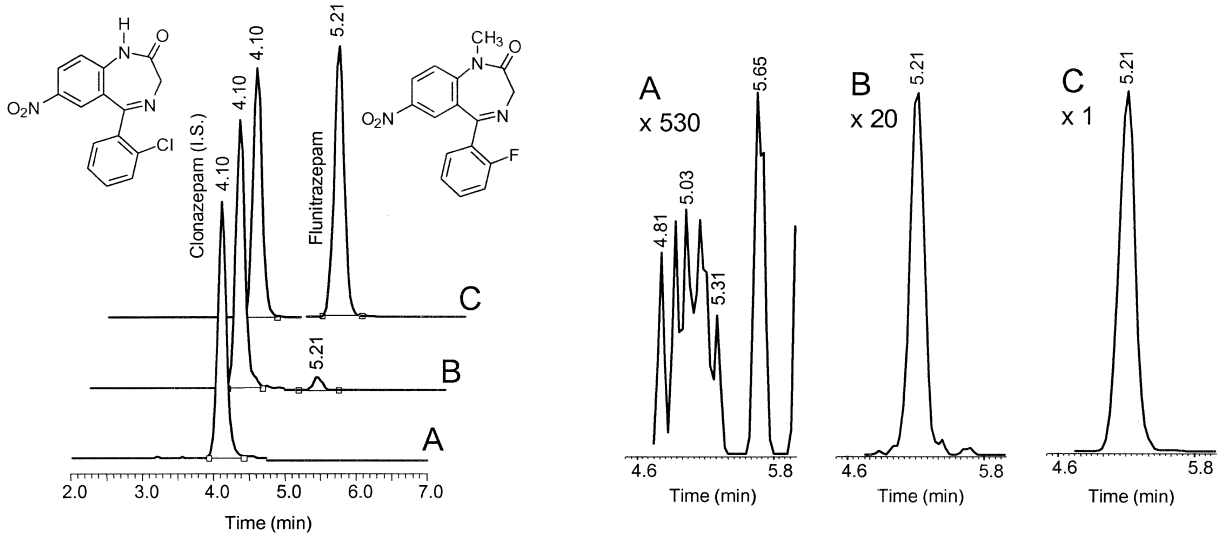


Fig. 1. Product ion chromatograms of calibration samples. The monitored product ions were *m/z* 270 for clonazepam (I.S.) and *m/z* 268 for flunitrazepam. (A) Blank serum sample with I.S.; (B) calibration sample 0.8 ng FNZ/ml; (C) calibration sample of 16 ng/ml of FNZ. On the left side all chromatograms are plotted on the same intensity scale and on the right side the time region of the FNZ peak is enlarged.

Table 1
Validation data

| | FNZ | | NZP | |
|---|---|---|---|---|
| Level (ng/ml) | 0.8 | 16.0 | 10.0 | 100.0 |
| *N* (number of samples) | 10.0 | 10.0 | 5.0 | 5.0 |
| Repeatability (%)[a] | 6.8 | 2.9 | 1.5 | 0.6 |
| Accuracy (%) | +5.7 | −2.0 | +4.6 | +5.6 |
| Reproducibility (%)[b] | 18.5 | 3.7 | – | – |
| Slope reproducibility (%)[c] | 4.8 | – | – | – |
| LOD (ng/ml)[d] | 0.19 | – | – | – |
| LOQ (ng/ml)[e] | 0.5 | – | – | – |

[a] Within-day precision.
[b] Day-to-day precision.
[c] RSD of the calibration curve slope calculated from five-point calibrations on nine different days.
[d] Estimated from a ten-point calibration from 0.2 to 2 ng/ml, SNR≈7:1.
[e] RSD (0.5ng/ml)=10.7%, $N$=5.

ng/ml was about 7:1. The limit of quantitation was defined to be 0.5 ng/ml [RSD (0.5 ng/ml)=10.7%]. The validation data of our method are listed in Table 1. These results are comparable to those of Bogusz et al. [8]. They defined an LOD of 0.2 ng/ml by an SNR of 3:1 and achieved a reproducibility of 17% for an urine sample containing 1 ng/ml of FNZ [8]. The better SNR at a level of 0.2 ng/ml should be due to the second MS stage in our method. In contrast to the results reported from GC–MS–MS analyses [10], we proved that the ion trap is a suitable LC–MS–MS detector for benzodiazepines.

The direct transfer of an established HPLC–UV method to an LC–MS–MS version enables the use of the mass spectrometer as a second, independent, highly selective detector for NZP and CLN as well. This is of importance in cases where HPLC–UV

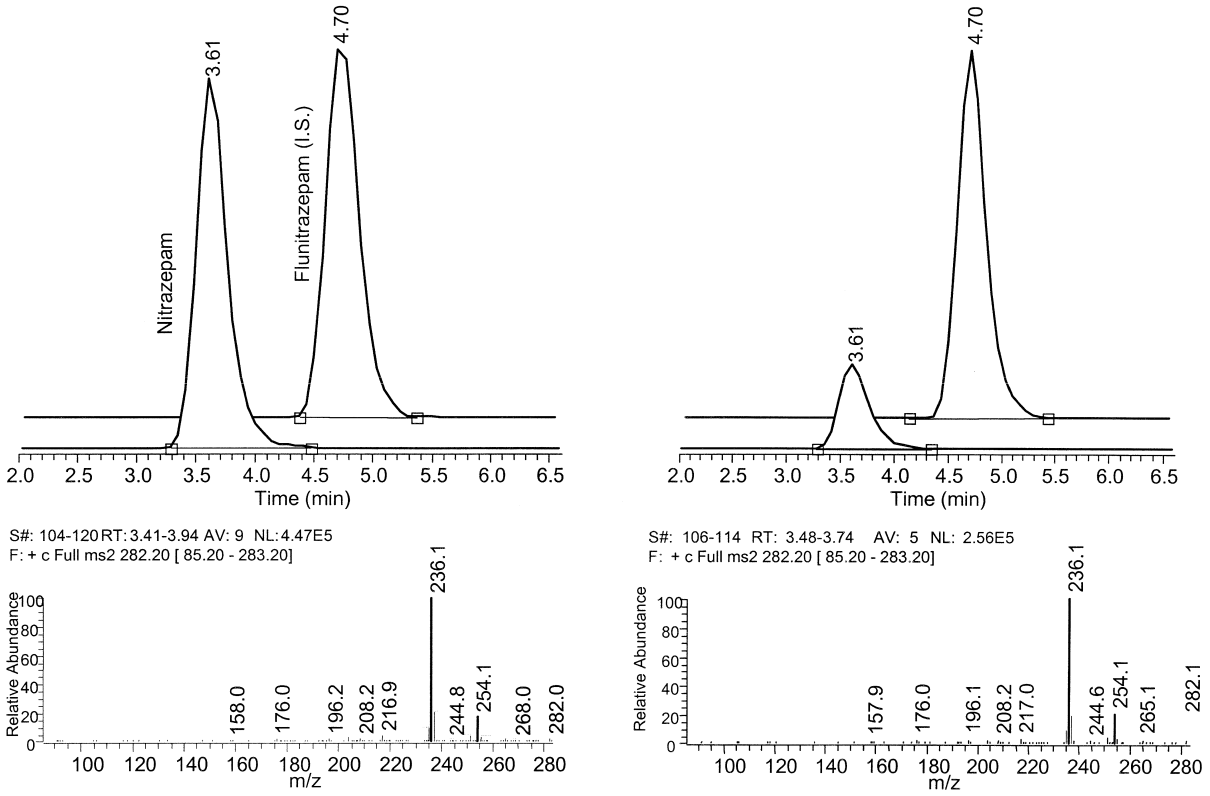


Fig. 3. Product ion chromatograms of nitrazepam samples (on the left is the patient sample and on the right is the calibration sample=100 ng/ml NZP). The plotted product ion traces are $m/z$ 236 for nitrazepam and $m/z$ 268 for flunitrazepam. Below each chromatogram the product ion spectrum within the nitrazepam peak is shown.

results should be confirmed (e.g., extremely high serum concentrations). Compared to a triple–quadrupole mass spectrometer the ion trap has the advantage that full-scan product ion spectra can be recorded without any loss of sensitivity. Fig. 3 presents an example of a serum sample with a high NZP concentration confirmed by an APCI–MS–MS chromatogram and the product ion spectra in the peak of interest.

## 4. Conclusions

Our validated HPLC–UV method for the determination of CLN and NZP using FNZ as the I.S. was easily transferable to an LC–APCI–MS–MS system enabling the quantitation of FNZ using CLN as the internal standard. The method features a very simple liquid–liquid extraction, the use of a standard 4-mm HPLC column, isocratic elution using a simple, buffer-free solvent, short retention times in connection with good peak resolution and, most importantly, the sensitivity and selectivity of an MS–MS detector.

The combination of HPLC and APCI–MS–MS using an ion trap detector has proven to be a valuable tool for the quantitative analysis of benzodiazepines in human serum and applicable to the therapeutic drug monitoring of flunitrazepam and related benzodiazepines.

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