# GC–MS Determination of Flunitrazepam and its Major Metabolite in Whole Blood and Plasma

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## Abstract

A gas chromatography–mass spectrometry method was developed for the analysis of flunitrazepam (FN) and its major metabolite, 7-amino-flunitrazepam (7-amino-FN), in both plasma and whole blood. The method was based on acid hydrolysis of the samples after dilution with HPLC water followed by extraction and derivatization (heptafluorobutyrate) of the resulting benzophenones. Analysis of plasma and whole blood samples from subjects administered 2-mg doses of FN showed that FN was only detected in whole blood (LOD 5 ng/mL) and not in plasma. However, 7-amino-FN was detected in both plasma and whole blood, although the levels were much higher in plasma. 7-Amino-FN was detected for the entire period of specimen collection (12 h), but FN was only detected in whole blood for 4 h after ingestion with peak levels after 1 h.

## Introduction

Flunitrazepam (FN), or Rohypnol®, is a sleeping aid that belongs to a group of newer-generation benzodiazepines that are administered in small dosage. Although it is not legally available in the United States, it has appeared on U.S. streets and been alleged to have been involved in some date-rape cases (1,2).

Studies (3,4) on excretion patterns of FN showed that 7-aminoflunitrazepam (7-amino-FN) is the most predominant metabolite in urine with the parent drug detected in only a few cases. The detection window of 7-amino-FN in urine can be extended to at least 72 h after ingestion (4). In the meantime, there are some questions about the concentration of FN and its metabolites in blood. It was reported (5,6) that the concentration of 7-amino-FN can greatly exceed that of unchanged parent drug in stored blood samples as a consequence of an in vitro reduction of the 7-nitro group of FN. In a previous communication (4), we reported on a sensitive gas chromatography–mass spectrometry (GC–MS) procedure for the analysis of FN metabolites in urine samples to detect prior ingestion of the drug.

Because serum or, more often, blood specimens are commonly collected for forensic toxicological analysis, this communication was initiated to present a simple extraction method and a sensitive GC–MS procedure for the detection of both 7-amino-FN and FN in both serum (or plasma) and whole blood and to ascertain the time course of detection of the parent drug versus its metabolite in both plasma and blood samples collected at the same time.
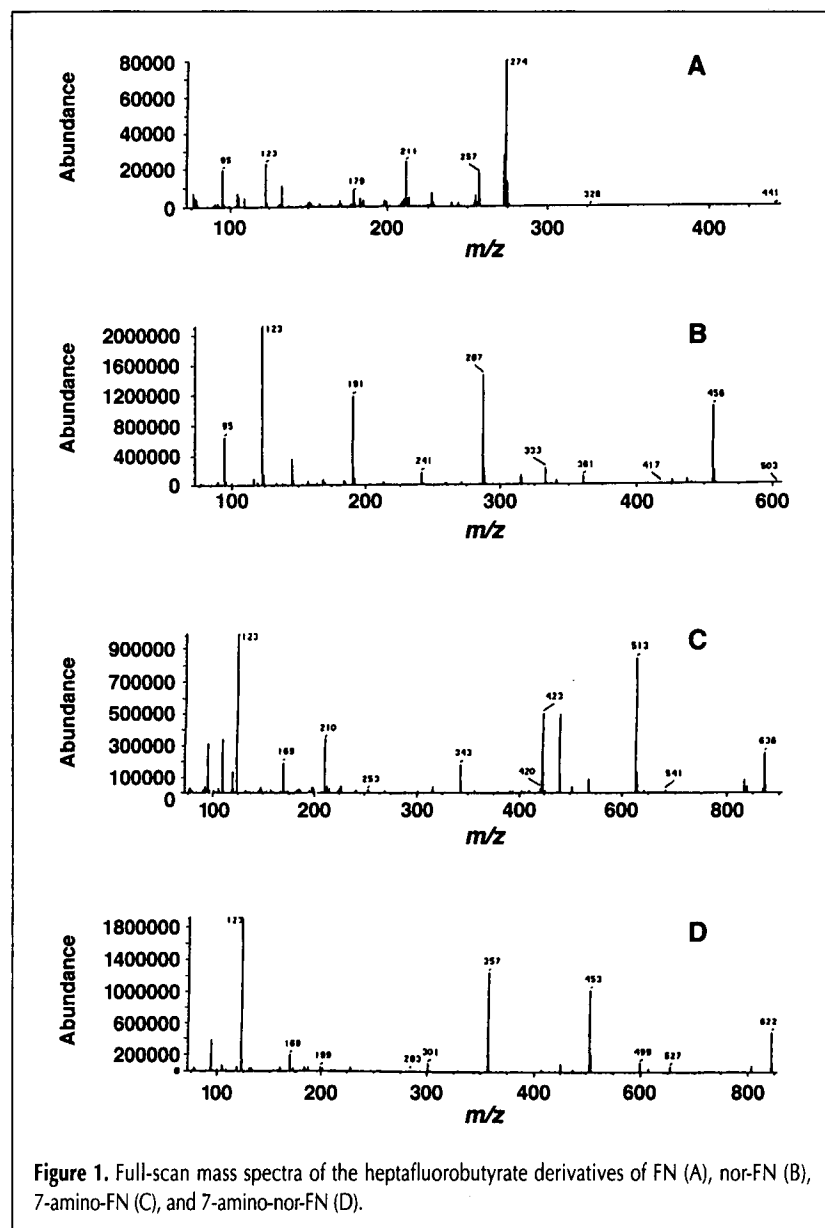
## Experimental

### Materials

7-Amino-FN-$d_3$ was purchased from Lipomed, Inc. (Cambridge, MA). FN, nor-FN, and 7-amino-FN were obtained from Radian Corp. (Austin, TX). 7-Amino-nor-FN was provided by Roche Diagnostics Systems (Somerville, NJ). All solvents were high-performance liquid chromatography (HPLC) grade, and the chemicals were American Chemical Society reagent grade.

### Extraction, hydrolysis, and derivatization

To 1 mL of plasma or blood sample in a 15-mL centrifuge tube, 25 µL of 7-amino-FN-$d_3$ at 1 µg/mL (25 ng/mL of plasma or blood concentration) was added. Three milliliters of water (HPLC grade) was used to dilute the sample, which was then vortex mixed briefly, followed by the addition of 1 mL of concentrated HCl. The tube was tightly capped and placed in an oven at 100°C for 1 h. After the tube was taken out of the oven and cooled to room temperature, 1.5 mL of 10N KOH was added, and the sample was vortex mixed. Six milliliters of chloroform was used to extract the sample by shaking gently for 2 min. After the sample separated into two layers (centrifugation may be needed to help phase separation), the top aqueous layer was aspirated to waste. The chloroform layer was washed with water twice (1 mL each) and then poured into a 13 × 100-mm test tube. The solvent was evaporated under $N_2$ at 50°C in a water bath. The residue was dissolved in 0.5 mL of chloroform containing 20 µg/mL of 4-pyrrolidinopyridine, and 100 µL of heptafluorobutyric anhydride was added. The sample was vortex mixed and allowed to stand at room temperature (65–75°F) for 1 h before the addition of 0.2 mL of 2N NaOH and 1 mL of 1.5M carbonate buffer (pH 11). After vortex mixing for 30 s, the aqueous layer was aspirated to waste. One milliliter of deionized water was added, and the sample was vortex mixed for 30 s. The organic layer was then transferred to a GC vial, and the solvent was evaporated under $N_2$ at 50°C. The residue was dissolved in 50 µL of ethyl acetate for GC–MS analysis. Two microliters of sample was injected.

**Figure 1.** Full-scan mass spectra of the heptafluorobutyrate derivatives of FN (A), nor-FN (B), 7-amino-FN (C), and 7-amino-nor-FN (D).

**Table I. Concentrations (ng/mL) of FN and Metabolites in Blood Collected from Two Subjects Administered a Single 2-mg Oral Dose**

| Subject | Time postdose (h) | FN | 7-Amino-FN | 7-Amino-Nor-FN | Nor-FN |
|---------|-------------------|------|------------|----------------|--------|
| A | 0 | 0.00 | 0.00 | 0.00 | 0.00 |
| A | 0.5 | 5.66 | 5.13 | 0.00 | 0.00 |
| A | 1 | 8.06 | 5.89 | 0.00 | 0.00 |
| A | 2 | 6.21 | 6.06 | 0.00 | 0.00 |
| A | 4 | < LOD | 6.35 | < LOD | 0.00 |
| A | 8 | < LOD | 5.78 | < LOD | 0.00 |
| A | 12 | < LOD | 5.62 | 0.00 | 0.00 |
| B | 0 | 0.00 | 0.00 | 0.00 | 0.00 |
| B | 0.5 | < LOD | 1.70 | 0.00 | 0.00 |
| B | 1 | 7.27 | 2.13 | 0.00 | 0.00 |
| B | 2 | 5.84 | 3.88 | 0.00 | 0.00 |
| B | 4 | 5.94 | 5.23 | 0.00 | 0.00 |
| B | 8 | 0.00 | 5.17 | 0.00 | 0.00 |
| B | 12 | 0.00 | 4.20 | 0.00 | 0.00 |

## GC–MS conditions

GC–MS conditions were similar to those used in the analysis of urine samples (4) but required a relatively longer run time for plasma or blood analysis. A Hewlett Packard (Palo Alto, CA) 5890 GC interfaced with a Hewlett Packard 5970 mass selective detector was used. The electron multiplier voltage was set at 200 V above the tune value. The GC was equipped with a 25-m × 0.2-mm (0.33-μm film thickness) DB-5 MS column operated in the splitless mode with purge valve closed for 0.2 min. Helium carrier flow was 36.2 cm/s under 25 psi column head pressure. Oven temperature was set at 180°C, held for 0.5 min, then increased to 260°C at 20°C/min, where it was held for 1 min, then finally increased to 280°C at 30°C/min, and held for 8 min. The injector and detector temperatures were 250 and 280°C, respectively. A Hewlett Packard G1034C version C.02.00 ChemStation data system was used to record the data. SIM ions for the various analytes were as follows (underlined ions are quantitation ions): 516 and 639 for 7-amino-FN-$d_3$ ($R_t$ = 6.30 min); 513, 636, and 439 for 7-amino-FN ($R_t$ = 6.32 min); 274 and 257 for flunitrazepam ($R_t$ = 8.53 min); 453, 622, and 499 for 7-amino-nor-FN ($R_t$ = 5.92 min); and 456 and 333 for nor-FN ($R_t$ = 6.20 min).

## Clinical studies

Four subjects were recruited for these studies with the proper consent forms and protocols approved by the institutional IRB.

Two clinical studies in which two subjects were administered a 2-mg oral dose of flunitrazepam were conducted. In the first study, blood specimens were collected from subjects A and B at 0, 0.5, 1, 2, 4, 8, and 12 h postingestion. In the second study, subjects C and D were each administered a 2-mg dose of flunitrazepam, and blood samples were collected at 0, 0.25, 0.5, 1, 2, 4, 8, and 12 h after dosing. However, in the second study, half of the blood samples were centrifuged and the plasma separated for comparison of the drug and metabolites level in plasma versus whole blood.

## Results and Discussion

In developing analytical methods for drugs and metabolites in plasma or whole blood, extraction procedures often involve protein precipitation with methanol or acetonitrile. In our efforts to develop a procedure for flunitrazepam and its metabolites, an initial protein-precipitation step was carried out using 3 mL of methanol per

milliliter of plasma or whole blood. This was followed by centrifugation, evaporation of the methanol, and acid hydrolysis of the extract. This extraction procedure was only partially successful because of the strong protein binding of the analytes. This resulted in inconsistent extraction efficiency, and in some cases, particularly with whole blood, neither the drugs nor the internal standards were recovered.

Other extraction methods such as liquid–liquid extraction using butyl chloride (6,7) and solid-phase extraction using Bond-Elut Certify® columns (8) or CleanScreen® extraction cartridges (9) produced unsatisfactory results for whole blood samples.

After several trials with different extraction conditions, a procedure was developed for both plasma and whole blood in which 1 mL (plasma or whole blood) was diluted with 3 mL of water (HPLC grade) first and hydrolyzed with hydrochloric acid directly without separation of the proteins. Most of the precipitate formed during the hydrolysis redissolved into the aqueous phase after basification. The analytes were then partitioned into $CHCl_3$. Washing twice with water resulted in a clean $CHCl_3$ extract. This process resulted in the formation of specific benzophenones corresponding to the individual drug and metabolites as previously described by ElSohly et al. (4). These benzophenones are then derivatized (heptafluorobutyrate) prior to analysis. Full scan mass spectra of the derivatized benzophenones corresponding to FN, nor-FN, 7-amino-FN, and 7-amino-nor-FN are shown in Figure 1.

The average recovery ($n = 5$) for 7-amino-FN and FN in whole blood and plasma was 93% and 53%, respectively, at 25 ng/mL This was determined by comparing the absolute peak area from extracted plasma specimens spiked with the analytes to the peak areas of the analytes spiked in HPLC-grade water at the same levels. The limits of detection (LOD) for 7-amino-FN, 7-amino-nor-FN, FN, and nor-FN in plasma were 1.0, 1.0, 5, and 5 ng/mL, respectively. The LOD was established as the lowest concentration of each analyte that provided a signal-to-noise ratio greater than 5 for the quantitation ion with the presence of the required confirming ions. The limit of quantitation (LOQ) for all analytes was found to be the same as the LOD.

All curves were linear up to at least 100 ng/mL. The precision of the method was determined at 5 ng/mL of all analytes, and the coefficient of variation ranged from 5.1% for 7-amino-FN to 11.7% for FN.

Figure 2 shows a typical chromatogram from a whole blood specimen analyzed by this method, with approximately 6 ng/mL 7-amino-FN and 8 ng/mL FN.

## Concentration of flunitrazepam and metabolites in whole blood

Clinical whole blood samples from two subjects

(A and B) administered a 2-mg dose of flunitrazepam were analyzed by this method. Table I shows that nor-FN and 7-amino-nor-FN were either too low to detect or not present. The method was able to detect the presence of 7-amino-FN at concentrations ranging from 1.70 to 6.35 ng/mL over a 12-h period with the peak concentrations appearing at 4 h. The parent drug, flunitrazepam, was also detected between 0.5 h to 4 h with the peak concentration appearing at 1 h in both subjects.
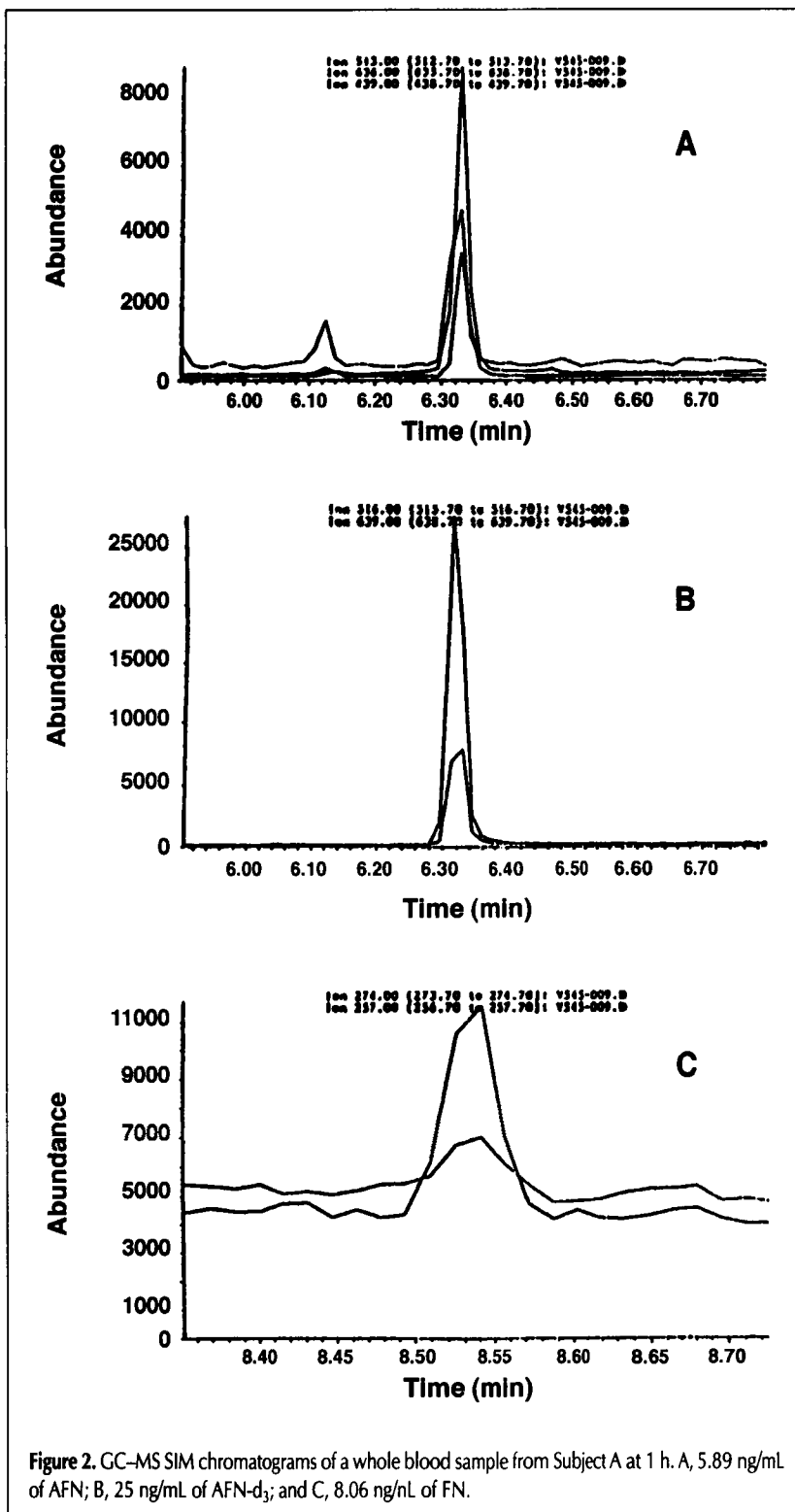


Figure 2. GC–MS SIM chromatograms of a whole blood sample from Subject A at 1 h. A, 5.89 ng/mL of AFN; B, 25 ng/mL of AFN-$d_3$; and C, 8.06 ng/nL of FN.

**Table II. Comparison of FN and 7-Amino-FN Concentrations in Blood and Plasma**

| Subject | Time (h) | Whole blood | | Plasma | |
|---|---|---|---|---|---|
| | | FN (ng/mL) | 7-Amino-FN (ng/mL) | FN (ng/mL) | 7-Amino-FN (ng/mL) |
| C | 0 | 0 | 0 | 0 | 0 |
| C | 0.25 | 4.60* | 0.95 | 0 | 2.16 |
| C | 0.5 | 5.70 | 1.49 | 0 | 4.11 |
| C | 1 | 6.77 | 2.83 | 0 | 10.4 |
| C | 2 | 4.64* | 2.38 | 0 | 12.2 |
| C | 4 | < LOD | 2.94 | 0 | 11.8 |
| C | 8 | < LOD | 2.93 | 0 | 5.21 |
| C | 12 | 0 | 3.16 | 0 | 6.81 |
| D | 0 | 0 | 0 | 0 | 0 |
| D | 0.25 | 0 | < LOD | 0 | < LOD |
| D | 0.5 | < LOD | < LOD | 0 | 8.11 |
| D | 1 | 3.47* | 1.00 | 0 | < LOD |
| D | 2 | < LOD | 1.67 | 0 | 7.58 |
| D | 4 | 0 | 2.31 | 0 | 10.3 |
| D | 8 | 0 | 2.80 | 0 | 4.21 |
| D | 12 | 0 | 2.80 | 0 | 6.74 |

*Lower than established LOD but quantitated because these samples showed acceptable ion ratios.

## Comparison of FN and 7-amino-FN concentrations in whole blood versus plasma in the same samples

Blood samples were collected from two subjects (C and D) at different times after oral ingestion of a 2-mg dose of flunitrazepam. Each sample was then divided into two portions.

One portion was kept as is and used for whole blood analysis. The second portion was centrifuged (1000 rpm) to separate the plasma which was analyzed for FN and 7-amino-FN. This experiment was carried out to determine the relative levels of FN and 7-amino-FN in plasma versus whole blood. We have previously observed (unpublished data) that FN was absent (below LOD of 10 ng/mL) in plasma samples.

Table II shows the concentration of FN and 7-amino-FN in whole blood versus plasma from the same samples. The data show that FN was present only in whole blood and not in plasma.

However, 7-amino-FN was present in both plasma and whole blood, although the levels were much higher in plasma, suggesting the predominance of the metabolite in plasma versus cells, possibly because of matrix effect.

The absence of FN from plasma samples in this study is not in total agreement with previous reports (10,11). This could be due to the differences in the sensitivities of the methods used (GC–electrochemical detection and liquid chromatography–mass spectrometry in the previous work with sensitivity of 0.2–1.0 ng/mL versus GC–MS at 5 ng/mL in this report). However, the blood levels found in this study were in agreement with those reported by Bogusz et al. (11) for the clinical samples.

## Conclusions

The GC–MS procedure described in this communication has a high sensitivity to detect flunitrazepam and its major metabolite, 7-amino-FN, in plasma and in whole blood. The parent drug, however, was only detectable in whole blood for approximately 4 h after ingestion of 2-mg doses of flunitrazepam, whereas 7-amino-FN was detectable for the entire period of collection (12 h). Therefore, for forensic purposes, detection of FN in whole blood would indicate recent ingestion of the drug, and 7-amino-FN could be monitored for a longer period of time.

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