Serum and Urine Concentrations of Flunitrazepam and Metabolites, after a Single Oral Dose, by Immunoassay and GC–MS

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Abstract

A clinical study was conducted to assess the ability of commercially available immunoassays to detect flunitrazepam (FNP) in plasma and urine samples and to compare the results with those obtained by gas chromatography–mass spectrometry (GC–MS). The clinical study consisted of four individuals (two male and two female) who had taken a single 2-mg dose of FNP. Serum was collected over a 48-h period and urine was collected over a 72-h period. The serum and urine samples were analyzed by the COBAS® INTEGRA Serum Benzodiazepines assay (SBENZ), the TDx serum and urine Benzodiazepines assay, and GC–MS. The GC–MS procedure was developed for analysis of FNP and metabolites in plasma and urine using an acid hydrolysis step resulting in the formation of specific benzophenones corresponding to FNP and its metabolites. The relative sensitivities of the assays for the detection of FNP and metabolites in serum and urine were GC–MS > SBENZ > TDx. The immunoassay results for serum samples showed peak concentrations of FNP metabolites at 8 h after FNP ingestion for three individuals and at about 1 h for the fourth individual. The GC–MS, SBENZ, and TDx urine immunoassays detected drug above the stated limit of detection (LOD) in 44, 41, and 35 serial FNP urine samples, respectively. FNP metabolites were detected in urine samples with all three assays for up to 72 h after a 2-mg dose. The improved detection rate with the SBENZ assay as compared to the TDx assay is likely explained by its higher cross-reactivity with the major metabolite, 7-amino-flunitrazepam (7-amino-FNP), and its lower LOD.

Introduction

Rohypnol® or flunitrazepam (FNP) is a fluorinated derivative of the benzodiazepine series. Rohypnol is not legally available in the United States, even for medical purposes, but it is an approved medication for sleep disorders in other parts of the world. Rohypnol has various street names, Rochies, Rophies, Roofies, and has been implicated in cases involving sexual assault. Rohypnol produces sedative effects; induces muscle relaxation and memory loss; decreases inhibitions; and impairs judgment, motor skills, and memory. Sedation occurs 20 to 30 min after administration of a 2-mg tablet and lasts for several hours thereafter. There are recent reports of increased abuse and illegal diversion of the drug into the U.S. (1). Flunitrazepam belongs to the potent group of benzodiazepines, which are therapeutically effective at low doses, making its detection more difficult. It is therefore important to select an immunoassay screening method that has the ability to detect low doses of this newly abused hypnotic drug. There have recently been new gas chromatography–mass spectrometry (GC–MS) methods developed for the detection of FNP and its metabolites in urine (2) and in whole blood or plasma (3). In addition there is a description of FNP excretion patterns in urine comparing the Abuscreen OnTrak and OnLine Immunoassays to GC–MS (4). In that study, urine samples collected following ingestion of a 4-mg dose of FNP did yield positive results in both the OnLine and OnTrak assays for at least 32 h after ingestion. After a 1-mg dose, positive specimens were detected only by GC–MS, but not by immunoassay.

The detection of FNP abuse in urine samples between 1 to 16 h postingestion of low doses of the drug requires the selection of an immunoassay with good cross-reactivity to FNP metabolites. This is illustrated in a most recent study (5) where an antibody was generated that had very high cross-reactivity to 7-amino-FNP allowing its detection following ingestion of 0.5 mg FNP.

Although there are controlled studies using GC–MS methods to detect positive specimens in whole blood, plasma, and urine (2,3), there are no available studies evaluating commercial immunoassays for the automated detection of FNP and metabolites in a blood component and urine collected in parallel from the same subject.

This study was conducted to compare two immunoassays, SBENZ and TDx, with GC–MS for the detection of FNP and metabolites in serum and urine following ingestion of a 2-mg dose of FNP.

Experimental

Biological samples

The flunitrazepam clinical study was approved by the Ethics Committee Faculty of Medicine at the University of Bern. All subjects that participated in the study were volunteers, naïve to the drug, and supplied informed consent. Two healthy male subjects (27 years, 62 kg in weight, smoker and 28 years, 82 kg in weight, non-smoker) and two healthy female subjects (both 29 years old, 55 kg in weight, and non-smokers) participated in the study. A single oral dose of 2 mg of FNP was ingested. Blood samples were collected before ingestion of FNP and at 1, 2, 4, 6, 8, 12, 15, 24, 36, and 48 h after ingestion. Blood was allowed to clot, and then serum was collected by centrifugation. All specimens from the study were frozen at –24°C within 12 h to eliminate any issue of sample instability. Urine samples were collected, and the volumes were measured before ingestion and then at 2, 4, 8, 12, 18, 24, 36, 48, 60, and 72 h after ingestion. Urine specimens were accurately timed and collected in polypropylene bottles without additives and immediately refrigerated. All specimens from the study were frozen at –24°C within 12 h of collection.

Materials

FNP, 7-amino-FNP, desmethylflunitrazepam (nor-FNP), and 7-amino-nor-FNP were supplied by Hoffmann-LaRoche (Nutley, NJ). 7-Amino-FNP-d_3 was purchased from Lipomed, Inc. (Cambridge, MA). TDx Benzodiazepines Serum reagents, limit of detection (LOD) 12 ng/mL, and calibrators and TDx Benzodiazepines U reagents, LOD 40 ng/mL, and calibrators were purchased from Abbott Diagnostics Division (North Chicago, IL) and used according to the manufacturer's package insert directions.

The SBENZ assay for the analysis of benzodiazepines in serum and urine, from Roche Diagnostic Systems, uses fluorescence polarization (FPIA) technology and was run on the COBAS INTEGRA analyzer. Calibration of the SBENZ urine and serum assays was performed using the Abuscreen OnLine Serum Benzodiazepine calibrators from Roche Diagnostic Systems and the Lypochek Benzo/TCA Control-Set B from Bio-Rad, (Anaheim, CA). The LOD for this assay was stated to be 3 ng/mL for serum and 7 ng/mL for urine.

Immunoassay analysis

The COBAS INTEGRA from Roche Diagnostic Systems, (Basel, Switzerland) and the TDx analyzer from Abbott Diagnostics (North Chicago, IL) were used according to the manufacturer's instructions.

GC–MS analysis

GC–MS analysis of FNP and metabolites in urine was performed using a recently published procedure with a reported LOD of 1 ng/mL (2), except that 7-amino-FNP-d_3 was used as internal standard. GC–MS analysis of FNP and metabolites in serum was performed for the total 7-amino metabolites using acid hydrolysis procedure as well as for the free 7-amino metabolite using direct extraction and analysis.

GC–MS procedure for analysis of total 7-amino-FNP metabolites. To 1 mL of serum sample was added 50 µL of 7-amino-FNP-d_3 at 1 µg/mL (50 ng/mL of serum concentration). Three milliliters of methanol was added and the sample was vigorously vortex mixed for 2 min. The sample was then centrifuged at 3000 rpm for 5 min. The supernatant was poured into a 15-mL tube, and the precipitate was discarded. The methanol was evaporated to a volume of less than 0.5 mL under a stream of N_2 at 50°C. Two milliliters of deionized (DI) water and 1 mL of concentrated HCl were added. 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 added, and the sample was shaken 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 1 mL of DI water 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 DI 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 70 mL of ethyl acetate for GC–MS analysis. Two microliters of sample was injected. This described procedure is a modification of the recently published GC–MS procedure for whole blood and plasma (3).

A Hewlett-Packard (Palo Alto, CA) 5890 GC interfaced with a Hewlett-Packard 5970 MSD 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 43 cm/min. 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 (quantitation ions are underlined): 516 and 639 for 7-amino-FNP-d_3 (R_t = 5.70 min); 513, 636, and 439 for 7-amino-FNP (R_t = 5.71 min); 274 and 257 for flunitrazepam

Table I. Concentration of Total 7-Amino-FNP and Free 7-Amino-FNP in Serum Collected from Four Subjects Administered a Single 2-mg Oral Dose

Subject sample	Time after ingestion (h)	GC–MS conc. (ng/mL) Total 7-NH_2-FNP	GC–MS conc. (ng/mL) Free 7-NH_2-FNP	TDx conc. (ng/mL)	SBENZ conc. (ng/mL)
A	0	0	0	0	0
A	0.5	0	0	0	0
A	1	0.8*	0.25	0	0
A	2	3.6	1.4	0	0
A	4	7.0	3.2	0	0
A	6	12.5	5.9	0	5.3
A	8	11.6	5.0	0	5.4
A	12	11.6	4.4	0	3.3
A	16	13.5	4.1	0	0
A	24	17.5	5.3	0	0
A	36	11.3	3.4	0	0
A	48	8.6	2.7	0	0
B	0	0	0	0	0
B	0.5	3.1	0.95	0	4.1
B	1	4.6	2.0	0	6.7
B	2	9.8	4.1	16.5	8.5
B	4	10.6	5.7	0	4.4
B	6	ND†	4.1	0	0
B	8	12.7	4.3	0	13
B	12	10.9	5.0	0	9.5
B	16	10.8	3.6	0	0
B	24	ND†	3.9	0	0
B	36	7.3	2.9	0	0
B	48	5.0	2	0	0
C	0	0	0	0	0
C	0.5	0	0	0	0
C	1	3.1	1.5	0	0
C	2	6.1	2.3	0	0
C	4	10.3	4.1	0	5.6
C	6	9.7	4.0	0	4.6
C	8	4.5	3.6	0	12.2
C	12	7.0	3.9	0	0
C	16	6.1	3.6	0	0
C	24	7.7	3.7	0	0
C	36	6.0	2.7	0	0
C	48	4.0	2.3	0	0
D	0	0	0	0	0
D	0.5	0.9	0	0	0
D	1	ND†	2.1	0	5
D	2	4.7	3.3	0	0
D	4	5.9	5.8	0	3.3
D	6	5.2	6.2	0	0
D	8	5.5	5.5	0	0
D	12	5.8	5.8	0	0
D	16	5.6	4.8	0	0
D	24	4.5	4.3	0	0
D	36	4.5	3.8	0	0
D	48	7.2	2.4	0	0

* Lower than established LOD but quantitated because these samples showed acceptable ion ratios.
† Not detected, possibly because of the low extraction recovery evidenced by low intensity of the internal standard peak; insufficient sample to reanalyze.

(R_t = 7.56 min); 453, 622, and 499 for 7-amino-nor-FNP (R_t = 5.32 min); and 456 and 333 for nor-FNP (R_t = 5.55 min).

Extraction and GC–MS procedure for analysis of free 7-amino-FNP in serum. To 1 mL of serum was spiked 10 µL of 7-amino-FNP-d_3 at 1 µg/mL. Four milliliters of methanol was added, and the sample was vigorously vortex mixed for 2 min. Then it was centrifuged at 3000 rpm for 5 min, and the supernatant was evaporated to about 0.5 mL. One milliliter of 40% phosphate buffer (pH 9.0) and 4 mL of ethyl acetate were added, the sample was shaken for 2 min, and the aqueous layer was discarded. The organic layer was shaken with 1 mL of 1N HCl for 2 min and the organic layer was discarded. To the aqueous layer, was added 0.7 mL of 2N NaOH and 3 mL of ethyl acetate. The sample was shaken for 2 min. The organic layer was evaporated under N_2 at 50°C. The residue was dissolved in 0.5 mL of $CHCl_3$ containing 20 µg/mL of 4-pyrolidinopyridine, and 100 µL of heptafluorobutyric anhydride was added. The sample was vortex mixed and allowed to stand at room temperature 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 1 min, the aqueous layer was aspirated and discarded. The organic layer was washed with 1 mL of DI water and then transferred into a GC vial. The solvent was evaporated under N_2 at 50°C and the residue was reconstituted with 50 µL of ethyl acetate. Two microliters of sample was injected. GC–MS conditions were as follows: The oven temperature was programmed at 200°C, held for 0.5 min, then raised to 270°C at 30°C/min where it was held for 6.5 min. A splitless injector was used with purge valve closed for 0.6 min. The electron multiplier voltage was set at 600 V above the tune value. The retention time for 7-amino-FNP-d_3 and 7-amino-FNP were 8.51 and 8.54 min, respectively. The SIM ions were 454 and 482 for 7-amino-FNP-d_3 and 451, 479, and 460 for 7-amino-FNP.

Results

Both physical drug effects and analytical drug excretion results for this study were recorded. A 2-mg dose of flunitrazepam had various effects on the four subjects as based on both self-reporting by the individuals and independent observation by one of the authors. Physiological measurements were not made. Subject A became somewhat drowsy at 1 h, but exhibited no problem with speaking, orientation, or coordination. He was in a deep sleep at 2 h, experienced intense hunger at 4 to 6 h, and was completely recovered at 12 h. Subject B exhibited a loss of coordination and fell into a deep sleep at 1 h. Decreased coordination remained at 4 h accompanied by signs of amnesia and difficulty in speaking. He recovered by 12 h except for the persistent amnesia. Subject C experienced great fatigue at 1 h. At 4 h she was still very tired and had orientation and speaking problems with evidence of some amnesia. At 12 h, she was still tired, but the other side effects were gone. Subject D became euphoric and experienced slight vertigo at 1 h. At about 1 h, 20 min, she reported being very tired. At 2 h, subject D was completely recovered, and at 4 h she was alert showing no residual effects.

GC–MS analysis of the serum samples was carried out

Table II. Concentration of FNP and Metabolites in Urine Collected From Four Subjects Administered a Single 2-mg Oral Dose

Subject sample	Time after ingestion (h)	GC–MS conc. (ng/mL) 7-amino-FNP	7-amino-NOR-FNP	FNP	TDx conc. (ng/mL)	SBENZ conc. (ng/mL)
A	0	0	0	0	46.6	< LOD
A	2	27.4	5	0	0	< LOD
A	4	45.4	3	0	0	9.3
A	8	218.7	27.2	0	74.2	69.7
A	12	276.4	39.7	36.5	97.6	112.6
A	18	127.7	25	17.7	52.2	52.7
A	24	101.1	25.2	0	44.2	48.5
A	30	83.6	25	0	49.6	39.2
A	36	72.9	29.6	0	0	24.8
A	48	126.3	60.6	0	59.3	52.9
A	60	61.4	40.9	0	48.9	28.1
A	72	100.6	77.3	0	60.3	52.4
B	0	0	0	0	0	< LOD
B	2	142.4	11.7	35.1	0	14.4
B	4	160.6	9.3	36.1	43.2	41.6
B	8	309.8	23.9	48	98.1	141.9
B	12	423.2	48.3	62.8	103.2	174.2
B	18	463.5	80.3	0	109.3	179.4
B	24	332.2	47.1	0	78	134.7
B	30	177.3	33.1	0	42.6	35.2
B	36	343.3	77.6	0	97.2	95.2
B	48	89.6	20.6	0	75.2	58
B	60	93.4	33.3	0	40.3	18.6
B	72	109.6	53.1	0	68.9	47.7
C	0	0	0	0	0	< LOD
C	2	47.7	0	0	59.4	11.5
C	4	40.0	1.0	32.0	0	11.8
C	8	226.2	26.0	73.5	74.6	83.2
C	12	279.5	54.5	112.5	84	110
C	18	98.9	22.0	54.0	46	40.8
C	24	138	40.5	0	56.7	62.7
C	30	57.9	18.1	0	0	19.3
C	36	74.8	32.6	0	52	23.5
C	48	53.2	30.2	0	43.9	26
C	60	81	53.1	0	46.6	42
C	72	36.2	37.4	0	46.8	20.8
D	0	0	0	0	0	< LOD
D	2	19.4	0	0	0	7.3
D	4	69.7	4.1	0	0	11.7
D	8	89.6	3.3	18	0	21.3
D	12	174.9	24.4	53.7	61.3	66.5
D	18	231.5	34.3	20.1	69.8	84.5
D	24	157.8	22.9	0	53.9	48
D	30	175.7	32.9	0	72.2	59
D	36	298.6	101.2	0	98.5	139.6
D	48	139.4	43.7	0	65.8	51.9
D	60	122.3	57.2	0	77.6	59.2
D	72	82.3	34.2	0	56.9	35.7

by two procedures. First an acid hydrolysis procedure that converts all metabolites to a single common benzophenone was utilized. For example, 7-amino-FNP, 7-acetamido-FNP, 7-NH_2-3-OH-FNP, and its glucuronide are all converted to the same benzophenone upon acid hydrolysis. Similarly, FNP, 3-OH-FNP, and its glucuronide are converted to the same benzophenone. The LOD (determined as the lowest concentration of each analyte that provided a signal-to-noise ratio greater than 5 for the quantitation ion with acceptable ion ratios), limit of quantitation (LOQ, determined as the lowest concentration at which all acceptance criteria were met, including quantitation values within ± 20% of the target concentration), and linearity were determined. The LODs for 7-amino-FNP and FNP were 3 ng/mL and 10 ng/mL, respectively. The LOQ for each analyte was the same as the LOD. Other metabolites were not spiked and all curves were linear up to 200 ng/mL.

Second, because only 7-amino-FNP was detected during the analysis of the total metabolites, analysis of the serum samples was also carried out for the free 7-amino-FNP. A liquid–liquid extraction procedure, which employed a back-extraction step, was used to clean up the sample extracts prior to derivatization. Similarly, with this procedure, the LOD, LOQ, and linearity were determined. The LOD and LOQ were both at 1 ng/mL (lowest concentration tested), and the calibration curve was linear from 1 to 50 ng/mL, the latter being the highest concentration tested. The extraction recovery of the free 7-amino-FNP was 96% at 1 ng/mL. This was determined by comparing the peak area from the extracted serum sample spiked with 7-amino-FNP to the peak area of 7-amino-FNP in the unextracted sample at the same concentration.

The results obtained with the GC–MS method for the detection of total FNP and 7-amino-FNP in serum were similar to those previously reported for whole blood and plasma (3). 7-Amino-FNP was detected as early as 0.5 h following drug ingestion and throughout the collection period (48 h), but the parent compound, FNP, as well as 7-amino-nor-FNP and nor-FNP were not detected in the samples. Table I shows the GC–MS analysis values for the total 7-amino-FNP in the individual samples collected from four subjects. The table also shows the values for the free 7-amino-FNP. It is clear that for the majority of the samples the amount of the total 7-amino-FNP was 2–3 times that of the free 7-amino-FNP, except for subject D for whom the values of the total and free were very close up to the 36-h collection time.

As shown in Table I, the immunoassays (primarily the SBENZ assay) detected cross-reacting benzodiazepines in serum between 0.5 and 12 h after ingestion of a 2-mg dose of FNP with the concentration values for the SBENZ assay ranging from 3.3 to 13.0 ng/mL. This assay had a reported LOD of 3 ng/mL and cross-reactivities of 60.8% for 7-amino-FNP, 38% for 7-amino-nor-FNP, and 48% for nor-FNP relative to nordiazepam at 100-ng/mL cutoff level (Roche Diagnostic Corp. Package Insert).

Only one sample tested positive by TDx. This assay had a reported 12 ng/mL LOD and 15% cross-reactivity to the major metabolite, 7-amino-FNP (Abbott Labs. Package Insert).

The SBENZ immunoassay showed serum peak concentra-

tions between 7 and 8 h for 3 individuals and 1 h for the fourth individual, with the maximum concentration being 13.0 ng/mL. Peak concentrations by GC–MS for the free 7-amino-FNP occurred at 4 or 6 h with a maximum value of 6.2 ng/mL. The peak value for total amount of metabolites calculated as 7-amino-FNP, however, was much more variable, occurring between 4 and 48 h and ranging from 7.2 to 17.5 ng/mL. The immunoassays detected up to 13 (38%) of the 37 samples considered positive by GC–MS (Table I).

FNP, nor-FNP, and 7-amino-FNP above the LOD of the GC–MS method were found in urine (Table II). Although the quantitative values for the immunoassays were generally lower than with the GC–MS assay, very similar excretion patterns were observed for all three methods (Figure 1). In addition all methods produced positive results throughout the entire 72 h collection period. The SBENZ and TDx assays detected 43 (98%) and 36 (82%) samples, respectively, above their stated LOD values. These samples were considered positive relative to the GC–MS results.

Discussion

Serum immunoassays are commonly used in emergency room settings to identify expected ingested substances and subsequently treat patients. Urine assays are commonly used to determine past exposure to a drug. In the case of serum, the SBENZ assay exhibited much better sensitivity than the TDx assay for the detection of the presence of FNP and metabolites, but only identified 14 (38%) of the 37 GC–MS-positive samples. The better detection rate of the SBENZ assay in comparison to the TDx assay is likely due to the lower LOD and the higher cross-reactivity to 7-amino-FNP of the SBENZ assay. The lower values observed with the immunoassays when compared to GC–MS for serum samples is mainly attributed to the lower level of FNP metabolites in serum coupled with the low cross-reactivity of the assay antibody to 7-amino-FNP. The GC–MS methods with better sensitivity and discriminatory power provide more reliable quantitative values.

The excellent correlation of excretion patterns obtained by immunoassay and GC–MS with the urine samples is shown in Figure 1 and attributed to the more reliable immunoassay results obtained with these samples that contain significantly more measureable FNP metabolites than those found in the serum samples. The much higher GC–MS values are attributed to the fact that acid hydrolysis converts all metabolites sharing the same basic nucleus to one common benzophenone. For example, 7-amino-FNP, 7-amino-3-OH-FNP, and its glucuronide as well as 7-acetamido-FNP, 7-acetamido-3-OH-FNP, and its glucuronide would all be converted to one common benzophenone characteristic for 7-amino-FNP. If only one metabolite is to be monitored by GC–MS, 7-amino-FNP, the predominant metabolite detected, should be selected.

Conclusions

This report provides a controlled study comparing immunoassays to GC–MS for the detection of FNP ingestion. Serum and urine samples collected from the same subjects following ingestion of 2 mg FNP were evaluated. GC-MS detected the presence of only 7-amino-FNP in serum. 7-Amino-FNP was the predominant metabolite in urine, but 7-amino-nor-FNP and FNP were also detected with FNP found in only a few samples. The immunoassays are much less sensitive to FNP metabolites with only the SBENZ assay being able to detect as many as 37% of the GC–MS positive samples. The relative sensitivities of the assays for the detection of FNP ingestion is GC–MS > SBENZ > TDx for both urine and serum samples. All three assays produce similar excretion patterns and can be used for the analysis of urine samples. In general, the GC–MS assay, which detects 7-amino-FNP in serum and urine, is the best method to determine if a subject has ingested a single dose of flunitrazepam.

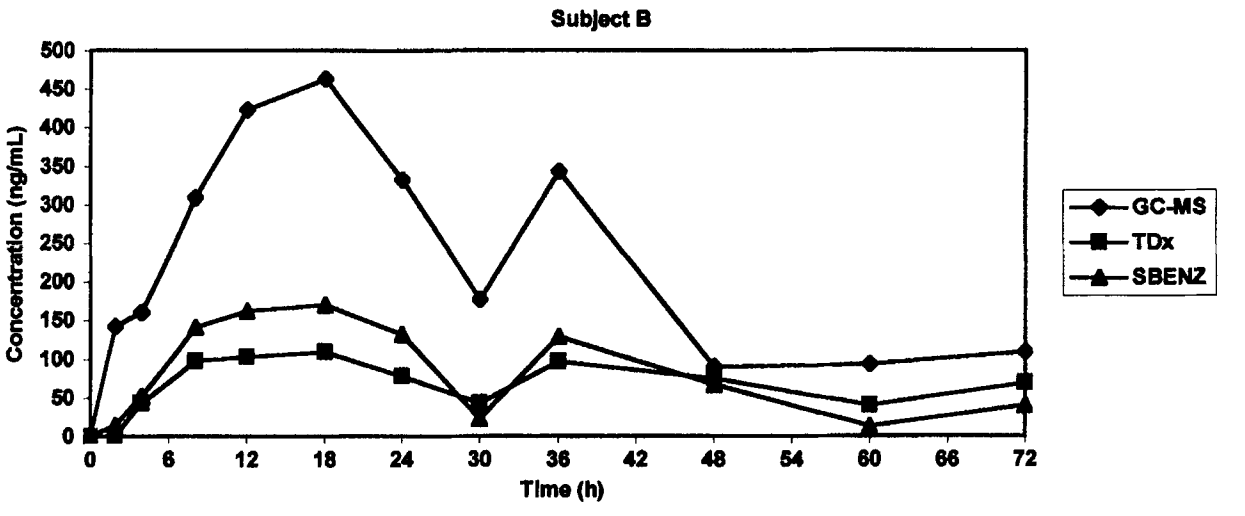

Figure 1. A typical urinary excretion profile from a subject (B) administered a single 2-mg dose of FNP. TDx and SBENZ immunoassay results (ng/mL) compared to 7-amino-FNP concentration (ng/mL) by GC–MS.

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