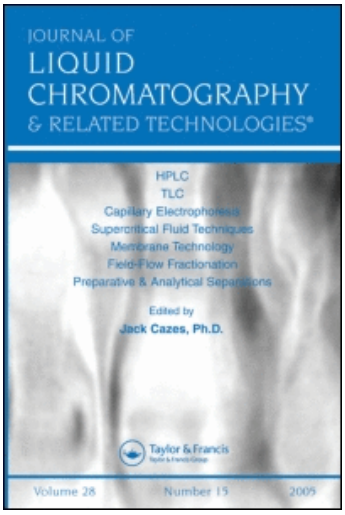


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# Considerations on MS/MS Detection of Bromazepam after Liquid Chromatographic Separation from Plasma Samples: Application to a Bioequivalence Study

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# Considerations on MS/MS Detection of Bromazepam after Liquid Chromatographic Separation from Plasma Samples: Application to a Bioequivalence Study

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**Abstract:** Bromazepam was isolated from plasma samples by simple protein precipitation with acetonitrile. A high throughput isocratic reversed phase separation between bromazepam and nitrazepam (IS) was achieved within 2 minutes on a rapid resolution cartridge column Zorbax SB-C18, using methanol/aqueous 0.1% formic acid 40/60 (v/v) as mobile phase. Detection was made by tandem mass spectrometry, using the multiple reaction monitoring mode (MRM). Both electrospray (ESI) and atmospheric pressure chemical (API) ionizations modes were considered, together with triple quadrupole (TQ) and ion trap (IT) mass analyzers. Ionization patterns are discussed; no major differences being observed between ESI and API. Higher sensitivity was obtained when using the TQ mass analyzer. Comparison between different ionization modes/mass analyzers types is made, based on bromazepam concentration/time profiles in real plasma samples. The best results were obtained in ESI/TQ conditions. Consequently, the method was fully validated, and then applied to a single dose (6 mg) open label, randomized, two period, two sequence, and crossover bioequivalence study of bromazepam in two commercially available tablet formulations. A low limit of quantification (LOQ) at 2.5 ng/mL level was obtained. Precision below 3% (expressed as relative standard deviation) and accuracy (expressed as % bias) within

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−7 and +11% were achieved. Pharmacokinetic parameters are presented, being in good accordance with literature data.

**Keywords:** API/ESI, Benzodiazepines, Extraction less sample preparation method, Human plasma, IT/TQ



## INTRODUCTION

Bromazepam (7-bromo-1,3-dihydro-5-(2-pyridinyl)-2H-1,4-benzodiazepin-2-one) is a psychotropic drug used for the short term treatment of major anxiety and temporary insomnia, as well as a sedative.[1] It represents an old, atypical congener belonging to 1,4-benzodiazepine class of compounds, most commonly prescribed in general medical practices.[2] Bromazepam binds to a ligand gated ion channel receptor ($GABA_A$) responsible for the mediation of $\gamma$-amino butyric acid, the major inhibitory neurotransmitter in the brain.[3] A real interest for bromazepam exists not only in clinical but also in forensic toxicology, as its prolonged use can lead to dependence, and acute overdoses, alone or in combination with alcohol or other depressants, and may produce coma or death.[4,5] The pharmacokinetic behavior of bromazepam is well studied. The biotransformation pathway involves hepatic microsomal oxidation, its clearance being significantly impaired in elderly individuals.[6,7] No significant changes in the pharmacokinetics and pharmacodynamics of bromazepam have been observed during co-administration with itraconazole and metoprolol.[8,9] Co-administration of cimetidine and propranolol reduces clearance and prolongs elimination half-life, especially in elderly individuals.[6]

Isolation of bromazepam from biological fluids was mainly achieved by liquid-liquid extraction (LLE).[10–14] Generally, better recoveries involve alkalinization of the aqueous phase. Diethyl ether, dichloromethane, chloroform, and mixtures of diethyl ether/n-alkanes or n-alkanes/ethyl acetate are more often considered as extractants. A sub zero temperature liquid-liquid extraction in acetonitrile was also reported in literature.[15] Solid phase extraction (SPE), carried out off- or on-line, has been used for isolation and concentration of bromazepam in human plasma and urine samples (metabolites in urine were enzymatically deconjugated).[16,17]

Gas chromatography has been used for separation of bromazepam against congeners and co-extracted matrix, the quantitative assay being achieved with electron capture detection (ECD), MS, or MS/MS.[18–24] Literature also cites quantitative thin layer chromatography for determination of bromazepam in biological fluids.[25] Undoubtedly, the reversed phase liquid chromatography (RPLC) separation mechanism represents the method of choice for determination of bromazepam in biological matrixes.[10–17,26–29] Very often, when using RPLC, the UV spectrometric detection in the 230–254 nm range was applied, allowing limits of detection (LOD) from 0.006 to 3 $\mu$g/mL.[10,11,13,15,16,26,28]

Increased sensitivity (2 ng/mL) was obtained when using reductive electrochemical detection.[13] MS/MS has been reported for selective and sensitive detection of bromazepam.[12,14,17,27,29] Both API[27,29] and ESI[12,14,17] were used as ionization modes. Resulting LOD values ranged from 0.3 to 1 ng/mL.

Bromazepam degrades in acidic conditions, leading to N-(4-bromo-2-(2-pyrydilcarbonyl)phenyl)-2-aminoacetamide.[30] After this first pH controlled, reversible step, the intermediate decomposes to 2-amino-5-bromophenyl-2-pyridylmethanone. The mechanism involves the cleavage of the 4,5-azomethine bond, followed by the breakage of the 1,2-amide bond.

Bioavailability data for bromazepam in terms of plasma concentration indicates maximum values placed around the 80 ng/mL level. It became evident that, for an interval ranging from 2 to 100 ng/mL, there is no real need for applying complex LLE or SPE sample preparation procedures for sensitive detection techniques, such as MS/MS. The aim of the present work relates to development and validation of a method involving a simple and fast extraction less sample preparation procedure, followed by a high throughput RPLC separation and a sensitive MS/MS detection, used for bioequivalence studies of bromazepam in pharmaceutical formulations (solid oral dosage forms). As detection plays an essential role to ensure the required method's overall sensitivity, ESI, and API ionization sources, as well as TQ and IT mass analyzers, were considered for use during method development. The validated method was then applied to a bioequivalence study of two commercially available pharmaceutical formulations containing bromazepam. Calculated pharmacokinetic parameters and statistical interpretation are also provided.



## EXPERIMENTAL

### Instrumentation

Experiments were performed with an Agilent 1100 series liquid chromatograph (Agilent Technologies, Inc.) system consisting of the following modules: degasser (G1379A), binary pump (G1312A), thermostated autosampler (G1329A), and column thermostat (G1316A).

The ion trap mass spectrometric detector Agilent SL series (G24450) was used in combination with API (G1947A) and ESI (G1948A) ionization sources. System control and data acquisition were made with the Agilent LC/MSD trap software version 4.2, integrating the MSD Trap Control software version 5.2. from Brucker Daltronics. The Agilent triple quadrupole mass spectrometric detector model G2571A was used together with the ESI (G1948B) ionization source and controlled over Agilent Mass Hunter Workstation Software version B.00.01. The systems had valid operational qualification status on use. The TQ instrument was operationally qualified before and after the bioequivalence study. The RSD values for reserpine

peak areas corresponding to 500 fg of the compound loaded to the ionization source vary from 3.9 to 13.7% over the study (including method validation period).

For ESI sources, the drying gas temperature was 350°C at 10 L/min flow. The pressure of the nebulizer gas was set at 60 psi. The capillary voltage was 4000 V when TQ is used as a mass analyzer. Optimized voltages of 2535 V for bromazepam and 2140 V for IS were used when the mass analyzer is the IT. For the API source, the following parameters were applied: vaporizer temperature: 350°C; drying gas temperature: 350°C; drying gas flow: 5 L/min; pressure of the nebulizer gas: 60 psi; corona voltage: 4000 V; capillary voltage: 3000 V.

Tandem mass spectrometric detection was used in the positive, multiple reactions monitoring (MRM) modes. Precursor ions are $m/z = 316/318$ a.m.u. for bromazepam and $m/z = 282$ a.m.u. for IS. Product ions are $m/z = 182$ a.m.u. for the analyte and $m/z = 180$ a.m.u. for the IS. For TQ detection, nitrogen is used as the collision gas. Collision energy was set at 35 V. For IT detection, collision gas is helium and the fragmentation amplitude 1 V. Wider precursor ion isolation windows were used in both cases.



## Chromatographic Methods

A rapid resolution cartridge Zorbax SB-C18, 30 mm L × 2.1 mm i.d., 3.5 μm d.p. fitted with a Phenomenex Guard Cartridge C18, 2 mm × 4 mm was used for the bioequivalence study. The column was validated before and after study completion, by computing the reduced plate height value ($\bar{h}$) for the fluoranthene peak (a variation from 3.1 to 3.9 was noticed during the whole process, meaning around 1100 injected samples). The column was thermostated at 25°C.

Isocratic elution was applied, using a mobile phase containing 60% aqueous 0.1% (v/v) HCOOH solution and 40% methanol, at a flow rate of 0.8 mL/min. Injection volume was 5 μL.

For applications using IT detection, it was necessary to replace the Zorbax SB-C18 cartridge column with a Purospher® Star RP-18e, 12.5 cm L × 4 mm i.d., 5 μm d.p., to allow higher injection volumes (200 μL). Mobile phase composition was also modified (56% aqueous component and 44% organic component represented by 10% acetonitrile in methanol), in order to generate similar capacity factors as for the experiments using TQ detection.

## Materials

All solvents were HPLC grade from Merck (Darmstadt, Germany). Water for chromatography (resistivity minimum 18.2 MΩ and TOC maximum 30 ppb)

was produced within the laboratory by means of a TKA Lab HP 6UV/UF instrument and used during experiments. Bromazepam and nitrazepam (IS) as standard reference substances were purchased from European Pharmacopoeia, Council of Europe, Strasbourg, France (bromazepam, cat. no. EPB1143000, batch 3j, and nitrazepam, cat. no. EPN 0900000, batch 1j).

## Sample Preparation Procedure

The IS solution of 400 μL in acetonitrile (50 ng/mL) were added to 200 μL of the plasma sample. A vortexing period of 5 min. at 2000 rpm was followed by centrifugation during 5 min. at 25°C and 7250 × g. An aliquot of the supernatant was transferred to a vial. When using IT detection, the supernatant was quantitatively collected, mixed with 400 μL of water for chromatography, vortexed for 5 min. at 2000 rpm, and transferred to a vial.



## Methodology and Pharmacokinetic Parameters

In this open label, randomized, two period, two sequence, crossover study, 24 healthy Caucasian volunteers (male/female ratio = 18/6) with a mean age of 21.6 ± 3.7 (18 ÷ 36) years, and a mean body weight of 67.3 ± 11.2 (46 ÷ 89) kg received two doses of 3 mg bromazepam from the tested product (T) and two from the reference product (R), in the sequence determined by randomization, with a 7 days wash out period between consecutive administrations. The protocol of the study was formally accepted by the evaluation department of the Romanian National Drug Agency and received the approval of the Institutional Ethics Committee. Venous blood samples were collected predose (0 h) and the following postdose intervals of time: 0.5; 1; 1.5; 2; 2.5; 3; 3.5; 4; 6; 8; 10; 12; 24, and 48 h. Medical examinations were performed in the screening and at the beginning of each study period (inhouse day), in every single blood sampling day, and at the end of each study period. The pharmacokinetic parameters considered for evaluation of the bioequivalence between tested and reference products were the following: 1. major parameters (according to FDA and EMEA recommendations), $C_{max}$ – observed maximum plasma concentration; $AUC_{last}$ – area under plasma concentration/time plot until the last quantifiable value; 2. auxiliary parameters, $T_{max}$ – sampling time of the maximum plasma concentration; $t_{half}$ – terminal elimination half life time; $AUC_{total}$ – area under plasma concentration/time plot extrapolated to infinity. Pharmacokinetic parameters were determined by means of the Kinetica™ software (version 4.4.1.) from Thermo Electron Corporation, U.S.A. The analysis of variance was performed on the pharmacokinetic parameters. Then, the 90% confidence intervals of the pharmacokinetic parameters characterizing the tested/reference products were determined.

## RESULTS AND DISCUSSION

### Method Development

Logarithms of the partition coefficients between n-octanol and water (log P) can be used for the right choice of the internal standard, as they are acting as synthetic descriptors of the hydrophobic character. For most of the congeners belonging to benzodiazepine class, the experimental log P is available. Our target was to find an IS exhibiting almost the same retention behavior as bromazepam, in order to achieve a fast separation of both compounds in less than 2 minutes. Such IS should be chosen among clonazepam, nitrazepam, and flunitrazepam (log P of 2.41, 2.25, and 2.06, respectively, compared to 2.05 for bromazepam). Despite their similar log P values, other congeners such as oxazepam (log $P = 2.24$), alprazolam (log $P = 2.12$), lorazepam (log $P = 2.39$), temazepam (log $P = 2.19$), and triazolam (log $P = 2.42$) exhibit higher retention, such behavior being explained by considering their intrinsic acidic/basic character ($pK_a$) in relation to the pH in the mobile phase. Nitrazepam has been chosen for multiple reasons: similar retention allowing almost baseline separation against the target compound (chromatographic resolution of 1.4) and generation of a product ion ($m/z = 180$ a.m.u.) situated very close to the product ion used for bromazepam ($m/z = 182$ a.m.u.).

With respect to their mass spectrometric behavior, both compounds generate protonated molecular ions within the ion source (ESI or API). Further collisional induced dissociation of the molecular ions lead to product ions $m/z = 298/300$, $288/290$, $261/263$, 237, 209, 182 a.m.u. for bromazepam and $m/z = 254$, 236, 207, and 180 a.m.u. for IS. Fragmentation schemas are proposed in Figure 1.

It is obvious that the IT detector is less sensitive compared to the TQ one. Subsequently, the sample preparation method and the chromatographic separation were modified in order to obtain similar limits of quantification. We found it interesting to compare the results generated by the different detection modes (ESI/TQ; ESI/IT, and API/IT). Consequently, each sample from a single phase and one volunteer collected during the bioequivalence study were split in four aliquots. Aliquots were processed according to procedures described in the experimental section and measured as following: two sets of aliquots were independently quantified with ESI/TQ, one set by means of the API/IT and the last one by means of ESI/IT. Each of the four determination sets has its own calibration with spiked plasma samples and verification by means of the quality control (QC) samples. Conditions related to back interpolation of the calibration experimental values and QC samples were verified as for the real bioanalytical procedure. Correlations between the concentration values obtained for real plasma samples through the formerly described procedure are presented in Figure 2. Correlation between data obtained with ESI/TQ during different experimental sessions

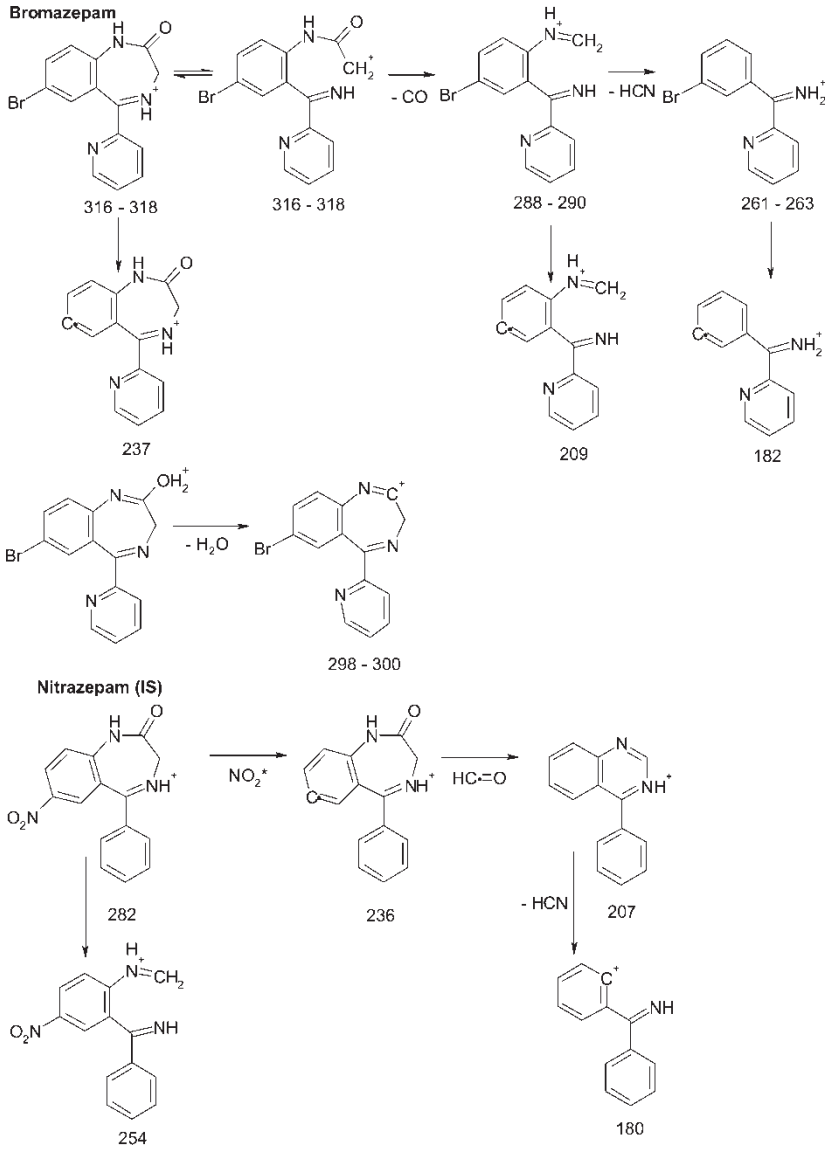


***Figure 1.*** Proposed CID ionization patterns for the target compound (bromazepam) and the IS (nitrazepam).

is excellent (with a correlation coefficient of 0.9958), proving an adequate precision. Good correlation was found between ESI/TQ and API/IT data ($r_{xy} = 0.9656$). A higher variability was observed when comparing ESI/TQ and ESI/IT experimental values ($r_{xy} = 0.8713$). The poorest correlation was

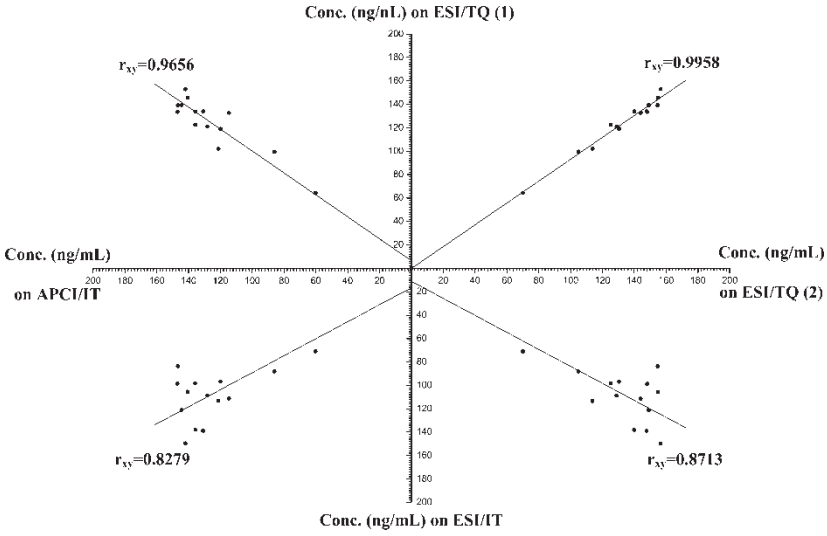


***Figure 2.*** Comparison between data obtained with ESI/TQ, API/IT, and ESI/IT detection modes on real samples obtained from one volunteer.

found for data issued from API/IT and ESI/IT experiments ($r_{xy} = 0.8279$). It seems clear that the right alternative to the ESI/TQ detection mode should be considered the API/IT design, when sample preparation is based on protein precipitation by means of organic solvent addition and no concentration step.

With respect to the separation conditions, it should be noted that retention of the analytes was characterized by capacity factors higher than 5 (more specifically, $k'$ was 7.2 for bromazepam and 11.3 for IS, respectively). Such retention behavior allows column effluent purging via the MS divert valve during the first 0.7 min of the chromatographic run, reducing the occurrence of the residual plasma matrix in the interface, and consequently, improving reproducibility on ionization. A typical chromatogram obtained with the ESI/TQ detector is shown in Figure 3. For illustrating method selectivity, a chromatogram of a blank sample was overlaid.

As the results obtained with ESI/TQ detection are the most reliable ones, the method was further considered for validation.

## Robustness

Retention of the target compounds varies non-linearly when the percentage of the organic solvent in the mobile phase ranges from 35 to 45%. The duration of the chromatographic run is halved when 45% of methanol is used in the mobile phase, while, at the other extreme of the interval, an increase of 25% of the time required for separation is observed. A $\pm 0.5\%$ modification

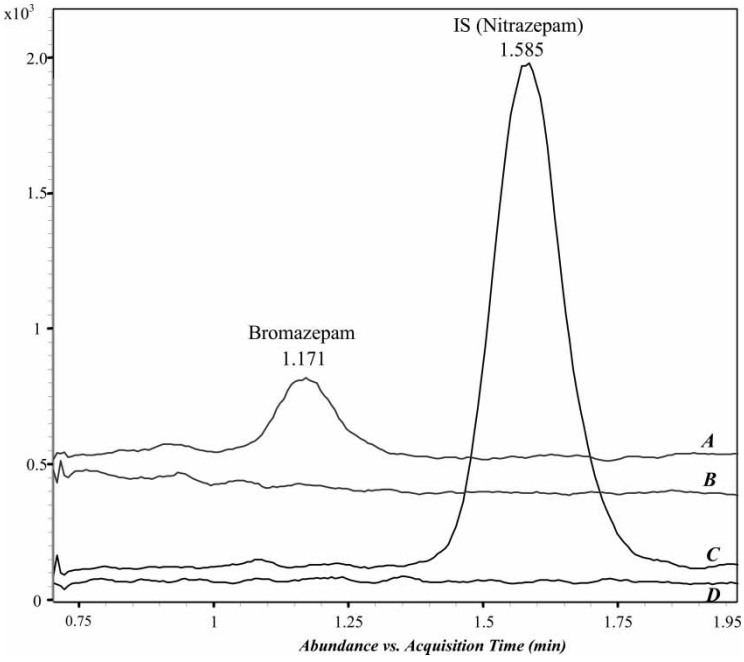


***Figure 3.*** Typical chromatogram obtained after method application on spiked plasma sample (trace A: bromazepam at LLOQ; trace B and D – blank sample; trace C: IS at 20 ng/mL level, meaning 1/5 from the concentration used in the method).

of methanol percentage in the mobile phase leads to retention data within the normal variation interval. Peak resolution is not extensively affected, variation between 1.2 and 1.7 being observed, although this parameter is not relevant (due to the ability of the mass analyzer to alternatively monitor both MRM transitions).

A higher content of formic acid used in the mobile phase slightly decreases retention and ionization yield. A 50% reduction and a 100% increase of the acidic additive in the aqueous constituent of the mobile phase still keep detector response within 15% precision and $\pm 5\%$ variation in retention of the analytes.

Column temperature variation of $\pm 1^{\circ}$C produces retention data within the normal variation interval. Variation of $\pm 5^{\circ}$C induces $\pm 10\%$ modification of the retention parameters, without critically influencing experimental results.

Columns belonging to three different production batches behave similarly on use.

No ionization effects were observed in relation with the type of anticoagulant used during the sampling procedure of the blood samples (lithium-heparin, ammonium-heparin, potassium edetate, and citrate were considered).

## Selectivity

Residual peak areas measured in blank samples over method validation and bioequivalence study completion vary from 1.3 to 9.1%, from the mean peak area produced by bromazepam in spiked plasma samples at low limits of quantification (LLOQ) level.

According to the bioequivalence study protocol, no comedication has been considered to avoid eventual potential adverse effects generated by the administration of the studied drug. None of the healthy volunteers involved in the study reports intake of concurrent medication during the testing periods. Despite the inherent selectivity induced by the detection process itself, some usual active substances have been considered for method selectivity evaluation. Plasma spiked samples at 1 μg/mL level, containing the following active products, acetylcysteine, acetylsalicylic acid, ascorbic acid, bromhexine, caffeine, chlorpheniramine, codeine, omeprazole, paracetamol, and ranitidine have been prepared and analyzed according to the method. No interference has been noticed.



## Recovery

Recovery of the target compound was studied on plasma samples spiked at three concentration levels (7.5 ng/mL; 60 ng/mL, and 120 ng/mL, respectively), compared to: a) corresponding spikes in protein precipitated bulk plasma sample (post-spikes); b) standard solutions made in the mobile phase. The concentration of the IS was 100 ng/mL. A mean recovery of 72.3% was found for bromazepam when compared to post-spikes, over the studied concentration interval and six individual trials at each concentration level (the relative standard deviation—RSD—of experimental recovery values was 3.6%). For the IS, the mean recovery was 72.5% with an RSD of 0.8% ($n = 18$). When compared to samples made in the mobile phase, mean recoveries are lower (62.9% for bromazepam and 62.5% for IS, respectively). This means that a reduction of the ionization yield of about 14% should be explained by the presence of the residual plasma matrix in the MS interface.

## Calibration and Limit of Quantification

The linearity interval of the detector response ranged from 2.5 to 200 ng/mL bromazepam in plasma samples. Six replicates were processed independently at each of the nine concentration levels (except zero). The linearity plot was expressed as peak area ratio between target compound and IS versus the concentration of bromazepam in spiked plasma samples. A slope, $B_1$, of $0.00898 \pm 0.00022$ and an intercept, $A_1$, of $-0.003 \pm 0.02$ were found as

parameters of the linear regression function. A correlation coefficient ($r_{xy}$) of 0.9994 has been computed.

Evaluation of the LOQ and subsequent calculation of the LLOQ = LOQ/2 and LOD = 0.33 × LOQ has been achieved by using the following relationship: $LOQ = [2 \times t \times (s_{A1} + s_{B1} \times C_{av})]/(B + 2 \times t \times s_{B1})$, where $s_{A1}$, $s_{B1}$ are the standard deviations calculated for $A_1$ and $B_1$, $C_{av}$ is the mean concentration value from the set used for the linear regression, and $t = 1.394$ is the student coefficient considered for $n - 2$ ($n = 10$) degrees of freedom and a confidence level of 90%. From the experimental dataset, the computed LOQ is 5 ng/mL. Accordingly, LLOQ should be considered at 2.5 ng/mL level and LOD around 1.6 ng/mL. These values are in good agreement with data resulting from evaluation of the signal to noise ratio in the corresponding chromatograms.

The RSD characterizing IS peak areas was 1.9% over the linearity procedure.

A calibration study was also made for the IS, to acknowledge that the planned concentration level spiked to plasma samples (100 ng/mL) fits within the linearity domain. The studied concentration interval ranged from 20 to 200 ng/mL of IS spiked to plasma samples. The linear regression ($y = A_2 + B_2 \times C$; y – peak area of the IS; C – concentration of IS spiked to plasma samples in ng/mL; $A_2 = -2908 \pm 3643$; $B_2 = 985 \pm 33$) was characterized by a $r_{xy}$ of 0.9991. LOQ is situated in the 10 ng/mL interval.

During study completion one calibration was achieved for samples obtained from three volunteers. Exception was made for the first and the last volunteer, each set of samples having its own calibration set. A total number of nine calibrations were consequently achieved over the whole study. Eight concentration levels were considered for the intra-sequence calibration (2.5; 5; 10; 25; 50; 75; 100; 150 ng/mL). None of the calibration samples failed to the back-interpolation checking operation. The slopes ($0.0087 \pm 0.0005$) computed for the calibration sets are characterized by an RSD of 2.9%.

## Precision

Precision was evaluated at three concentration levels (7.5, 60, and 120 ng/mL). The repeatability was carried out on 10 replicates of a single processed sample at each concentration level within the same experimental session. The intermediate reproducibility was studied on 6 spiked plasma samples at each concentration level, processed independently by different analysts in different days. Precision is described by the RSD calculated for the concentration data sets obtained by interpolation of the experimental peak area ratio in the linear regression equation. Repeatability is characterized by RSD values ranging between 1 and 2.9%. The normal variation intervals (mean $\pm 2 \times$ s) of the computed concentration values at each level were: $7.8 \pm 0.45$; $58 \pm 1.2$; $121.6 \pm 2.6$ ng/mL. Intermediate reproducibility is characterized by RSD

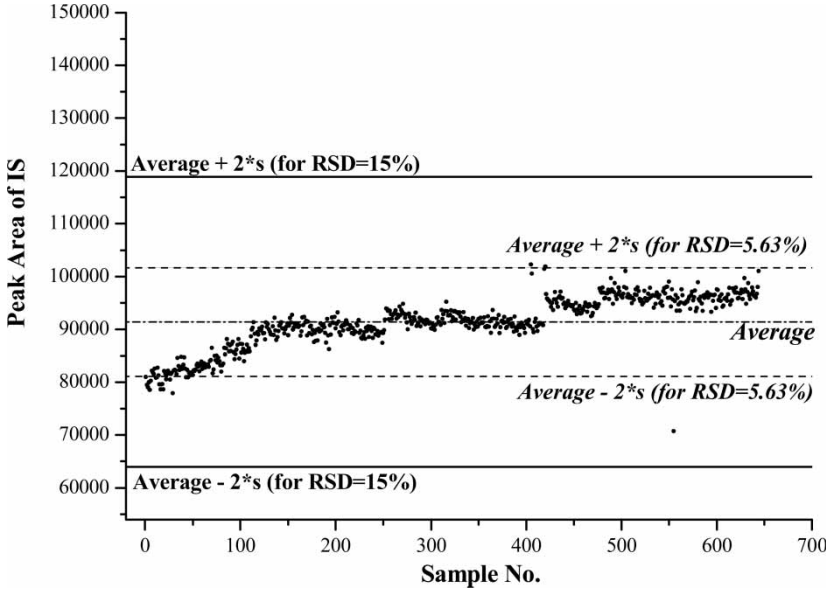


***Figure 4.*** Variation of peak area values for IS over the bioequivalence study completion.



values fitting within the 0.7 to 2.1% interval. The normal variation intervals for the concentration values were: $7.3 \pm 0.3$, $59 \pm 1.2$, $120 \pm 1.6$ ng/mL. One can conclude that most of the variability affecting experimental values is produced by the equipment and not by the sample preparation procedure. Within the experimental data sets, no evident variation trends were observed.

The population of peak areas values measured for the IS during study completion is characterized by an RSD of 5.6% (see Figure 4). The slightly positive trend observed in the variation of the IS peak area over the whole study may be explained by the accumulation of residual matrix in the interface (the system was operated over the study without cleaning of the interface).

## Accuracy

QC samples were injected during study completion. Spiked plasma samples at three concentration levels were considered: 7.5, 60, 120 ng/mL. Samples obtained from one volunteer were analyzed together with a QC set. Two QC samples were considered for each concentration level. Experimental concentration values were calculated by means of the calibration equations obtained during the corresponding analytical sequence. The bias observed

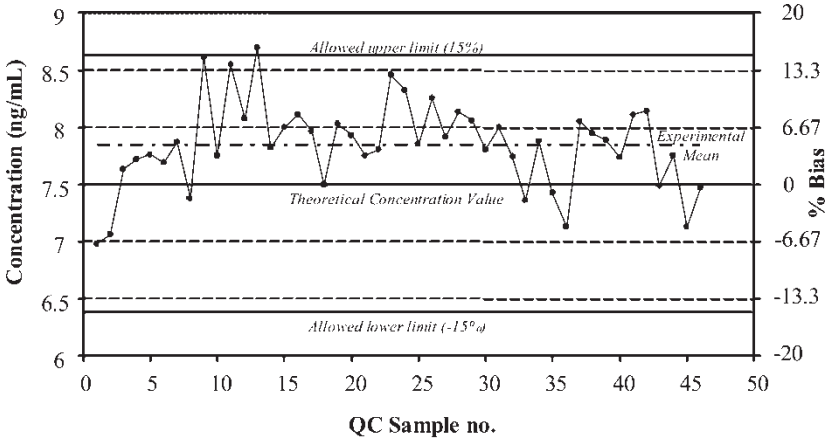


***Figure 5.*** Accuracy obtained for low concentration level QC samples during bioequivalence study completion.



between the experimental concentration value and the theoretical one (expressed as percentage) for each QC sample acts as an accuracy indicator.

The QC data obtained at the lowest concentration level ($3 \times$ LLOQ) should be considered as the critical one. Figure 5 illustrates variations of the experimental concentration values corresponding to the low level QC samples analyzed over the whole study ($n = 46$) and the corresponding % bias. A single value falls out from the acceptance interval ($\pm 15\%$). The experimental mean differs from the theoretical value by an absolute bias of 0.34 ng/mL, meaning that the bulk spiked plasma from which the low level QC samples originate was affected by an error of 4.5% on preparation. It is worthwhile to note that the linear regression obtained for the experimental concentration values is characterized by a correlation coefficient of 0.0028, a slope of $-0.0015$, and an ordinate to origin of 7.88. This suggests that experimental values are randomly positioned against the mean and consequently, no trend appears to affect the variation.

## Stability

The stability study for bromazepam was achieved at three different concentrations: 7.5, 60, and 120 ng/mL. Stability was concluded if the relative standard deviation calculated for concentration values determined for the analyzed samples is below 15%.

Freeze and thaw stability was determined for five consecutive cycles on spiked plasma samples. Determined RSD for the concentration data sets were 1.4% for the low level, 1.7% for the medium level, and 1.6% for the high level.

*Table 1.* Statistics of pharmacokinetic parameters

| Drug | Statistics | $C_{max}$ (ng/mL) | $T_{max}$ (hours) | $t_{half}$ (hours) | $AUC_{last}$ (ng/mL × h) | $AUC_{tot}$ (ng/mL × h) |
|---|---|---|---|---|---|---|
| Tested | Mean | 92.7 | 0.85 | 22.8 | 1955.2 | 2577.3 |
| (T) | RSD | 33.1 | — | 30.4 | 42.2 | 68.5 |
| Reference | Mean | 91.5 | 0.88 | 23.8 | 1953.2 | 2608.6 |
| (R) | RSD | 37.3 | — | 20.9 | 44.7 | 58.1 |
| 90% confidence interval for the ratio of the means T/R | | 89.3–115 | — | — | 91.3–109.8 | 90–108.5 |

Long term stability at −40°C was evaluated over a 60 days period. Samples were analyzed initially, after 20, 40, and 60 days. Determined RSD for the concentration data sets were 3% for the low level, 1.6% for the medium level and 1.6% for the high level.

Bench top stability was considered for a 24 hours period. Thaw samples were analyzed initially, and 2, 4, 12, and 24 hours latter. Found RSD values were, in the increased order of the sample concentrations: 3, 1.4, and 1%, respectively.

Post preparative stability (stability of the processed sample until injection) was investigated immediately after preparation, 1, 3, 6, 12, and 24 hours latter. Found RSD values were, in the increased order of the sample concentrations: 1.3, 0.7, and 1.5%, respectively.

The stability at room temperature of the IS stock solution in acetonitrile was also considered for a period of 4 days. Analyses were performed daily. The stock solution was added to blank plasma samples and prepared according to the experimental procedure. RSD was calculated on the peak area data set (4% value was found).

### Bioequivalence Data

From the total number of volunteers, 23 finalized the study. Pharmacokinetic data obtained on study completion are presented in Table 1. These data are in very good agreement with data presented in ref. [14] (the reference product was identical). From the analysis of the resulting data, it clearly results that additional sampling at 0.25 h and 72 or 96 h is strongly recommended.

## CONCLUSIONS

An extraction less sample preparation procedure is proposed for isolation of bromazepam in plasma samples. A high throughput chromatographic

separation has been achieved in isocratic conditions. The tandem mass spectrometric detection in the MRM mode was used. A parallel between results produced with ion trap and triple quadrupole designs was developed. Both ESI and API ionization sources were used. Best results in terms of sensitivity, precision, and accuracy are obtained on ESI/TQ instrumentation. When using IT mass analyzer, the API ionization source provides more reliable results compared to ESI. Collision induced dissociation (CID) ionization patterns are discussed for both analyte and IS. The HPLC-ESI/TQ method was fully validated according to ref. [31] (www.fda.gov/cder/guidance/index.htm) guidelines and used for a bioequivalence study. Pharmacokinetic results were in very good agreement with data in literature, produced by means of liquid-liquid extraction sample preparation and RPLC-ESI/MS/MS. Pharmacokinetic data sustained bioequivalence between the tested and the reference products considered for the study. Method sensitivity (LLOQ $\sim$ 2.5 ng/mL), precision, and accuracy were found suitable for bioequivalence purposes.



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