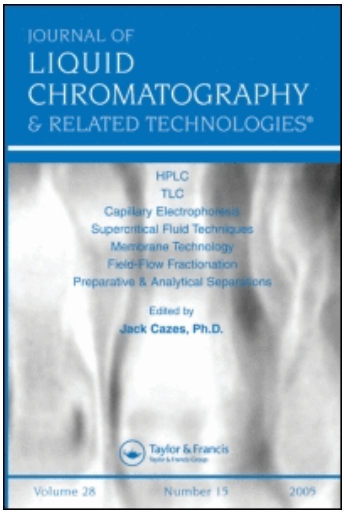

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Development and Validation of an HPLC Method for the Simultaneous Monitoring of Bromazepam and Omeprazole

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Abstract: Bromazepam and omeprazole are frequently administered to hospitalized patients to decrease anxiety and prevent stress ulcers, respectively. In patients under enteral nutrition, these drugs are administered via a nasogastric feeding tube. However, this mode of administration renders their bioavailability highly variable, and calls for their therapeutic monitoring. Given the absence of published data on the compared bioavailability of the two drugs given orally or via a nasogastric feeding tube, we have developed a high performance liquid chromatography method with UV detection (HPLC-UV) suitable for their simultaneous monitoring in human plasma. The method involves solid phase extraction of 2 mL plasma samples. Linearity was demonstrated in a concentration range of 5–100 ng/mL and 20–2000 ng/mL for bromazepam and omeprazole, respectively. The lower limit of quantification was 5 ng/mL and 20 mg/mL for bromazepam and omeprazole, respectively. The method proved its worth in the simultaneous monitoring of bromazepam and omeprazole administered to healthy volunteers.

Keywords: Omeprazole, Bromazepam, Therapeutic drug monitoring, Solid phase extraction, HPLC

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INTRODUCTION

In hospital practice, patients fed enterally are often polymedicated. A recent retrospective study in our University Hospital has shown that the drug treatment of many of these patients includes a benzodiazepine to decrease anxiety and a proton pump inhibitor to decrease the risk of gastric ulcers. The most frequently prescribed drugs in these two classes are bromazepam and omeprazole, respectively, and their usual mode of administration was by a nasogastric tube.[1,2]

Benzodiazepines are used as anxiolytics, anticonvulsants, sedatives, and myorelaxants.[3,4] The marked anxiolytic effect of bromazepam (Figure 1) accounts for its broad prescription to hospitalized patients, in which anxiety is frequently observed.[5–7] As for omeprazole (Figure 2), its main therapeutic indications are gastric and duodenal ulcer, reflux oesophagitis, and symptomatic gastro-oesophagal reflux.[8,9] In association with an antibiotic such as amoxicillin or clarithromycin, it is also used to eradicate the ulcer causing Helicobacter pylori.[10] In patients in intensive care, surgery, otorhinolaringology, and oncology units where anxiety[5–7] and risk of ulcer are greatest, it is prescribed mainly as a prophylactic in association with bromazepam.[11,12] Frequently, such patients may suffer from a non-functional gastrointestinal tract and must be fed by a nasogastric tube.

The administration via the nasogastric tube of drugs usually given orally presents a significant risk of highly variable bioavailability due to functional perturbations of the gastrointestinal tract, altered physiological conditions, and major differences in nutrients. Furthermore, the many and significant gaps in the relevant medical and pharmaceutical literature make therapeutic drug monitoring a necessity to assess and, hopefully, confirm bioequivalence in nasogastric tube administration. Our long term objective is, thus, to compare the bioavailability of orally versus nasogastrically administered bromazepam and omeprazole and, more generally, to gain a better understanding of the value and limits of nasogastric drug administration. A second objective

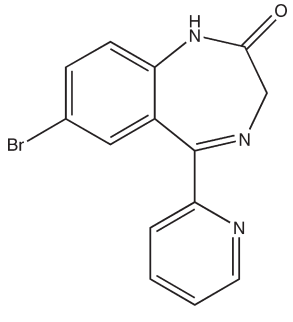

Figure 1. Chemical structure of bromazepam.

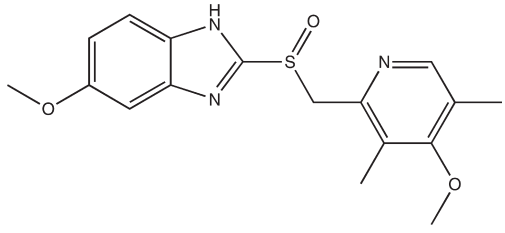

Figure 2. Chemical structure of omeprazole.

is to rule out a possible physicochemical incompatibility between drugs and the polyurethane material of the nasogastric tube.

Bromazepam and omeprazole have been the objects of a number of papers reporting their analysis in a variety of matrices,[13–18] none of which examined their simultaneous monitoring in biological matrices. In line with our long term objectives, we thus undertook to develop and validate a quantitative HPLC-UV method, allowing the simultaneous and fast monitoring of bromazepam and omeprazole in human plasma. A first application in a healthy volunteer is also reported, suggesting a good bioavailability via nasogastric administration and the absence of any incompatibility with polymeric material.

EXPERIMENTAL

Chemicals and Reagents

Pharmaceutically pure omeprazole was obtained from Sigma-Aldrich (Buchs, Switzerland). Bromazepam and the internal standard oxazepam[19] (Figure 3) were obtained as reference chemicals from the European Pharmacopea Commission (Europe Council, Strasbourg, France). Methanol (MeOH), acetonitrile (ACN), and water, all of LiChrosolv® HPLC quality, were bought from Merck KGaA (Darmstadt, Germany), as were potassium hydrogenophosphate LiChropur® (K_2HPO_4) and phosphoric acid 85% Suprapur® (H_3PO_4). Triethylamine

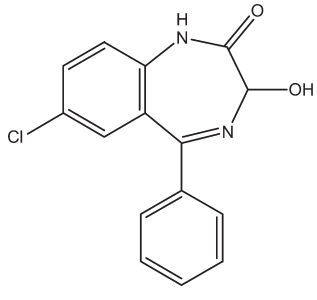

Figure 3. Chemical structure of oxazepam.

(TEA) ($C_6H_{15}N$) "99.5% for amino acid analysis" was obtained from Fluka (Buchs, Switzerland).

Samples of human plasma used as the model of the biological matrix were obtained from the blood transfusion unit of our University Hospital. Seven plasma samples with different characteristics (i.e., normal, lipemic, or obtained from hemolyzed blood) were used.

Instrumentation

The following instruments were used: a Mettler-Toledo AT 250 balance with a Mettler-Toledo LC-P45 Printer (Uznach, Switzerland); a Mini Vortexer® from VWR (Dietikon, Switzerland); a Rotanta RP centrifuge from Hettich (Baech, Switzerland); an ultrasound bath Bransonic® 5200 from Branson (Danbury, USA). The pH was measured with a Metrohm system (Herisau, Switzerland) verified and calibrated daily, composed of a 713 pH-meter, a LL-Unitrode® PT 1000 electrode, and a magnetic stirrer. Temperature sensitive products were stored at $-20^{\circ}C$ in a Luxus deep freezer from Bosch (Geroldswil, Switzerland). Storage at $-80^{\circ}C$ was in an Ultima® II deep freezer from Revco (Asheville, USA). The temperature in both deep freezers was monitored continuously by a link to the Technical Control Centre of our Hospital. The quantitative determinations were carried out with a Varian HPLC system (Palo Alto, USA) equipped with a Prostar® 230 pump, a ProStar® 410 automatic injector, and a UV Prostar® 310 detector.

Preparation of Solutions

An initial 1 mg/mL solution of bromazepam was diluted with methanol to a concentration of 2 μg/ml; 3 mL vials of this solution were stored at $-20^{\circ}C$.[18] An initial 2 mg/mL solution of omeprazole was diluted with methanol to a concentration of 40 μg/mL; 3 mL vials of this solution were stored at $-80^{\circ}C$, given the known lability of the drug.[20] The initial solution of internal standard was 50.0 mg oxazepam in 100 mL of methanol/H_2O (1:1 v/v); 3 mL vials of this solution were stored at $-20^{\circ}C$.[21]

The calibration and quality control standards were prepared directly on the extraction columns by mixing 200 μL of the test solution, 100 μL of internal standard solution, and 1.8 mL plasma.

Sample Preparation

The samples were prepared for quantitative analysis by solid phase extraction using Bond Elut Certify® columns (3 mL/130 mg) from Varian (Palo Alto,

USA), which comprises a packed bed consisting of a non-polar C_8 sorbent and a cation exchange phase SCX. Samples for analysis were thawed in the morning. The extraction column was conditioned with 1 mL methanol and 1 mL H_2O, followed by loading with 2 mL of plasma sample and 100 μL of internal standard. The column was washed with 1 mL H_2O and 1 mL acetonitrile 10%. Elution was carried out with 1 mL methanol under light vacuum. The eluate was evaporated under N_2 flux, reconstituted in 150 μL mobile phase and placed in an HPLC vial. The yield was always >95%.

Chromatographic Conditions

HPLC analysis was carried out by inline coupling of two different columns to improve resolution, namely a Chromolith™ Performance RP-8 column followed by Chromolith™ Performance RP-18 column (both 4.6 × 100 mm). This system was fitted with a Chromolith™ Performance RP-18 (4.6 × 10 mm) precolumn from Merck KGaA (Darmstadt, Germany). The mobile phase (acetonitrile/aqueous phosphate buffer 0.07 M, 19/81) contained 0.4% triethylamine and was adjusted to pH 7.4. Before mixing, the aqueous buffer was filtered on a GH Polypro filter (47 mm, 0.2 μm) from Pall (New York, USA). The flux was 3.5 mL/min for an injection volume of 50 μL. The detection of bromazepam, omeprazole, and oxazepam were set at 230, 302, and 230 nm, respectively. An automatic change in wavelength was programmed 8 and 12.5 min after injection, with each change being followed by an autozero resetting. Under such conditions, the retention times of bromazepam, omeprazole, and oxazepam were 7.2, 9.4, and 17.9 min, respectively. Before each series of analyses, 3 samples (working solution, mobile phase, and internal standard) were successively injected to check the operating conditions.

All analyses were carried out in a laboratory with temperature control (20–24°C).

Method Validation

Method validation was carried out according to the Guidelines of the French Society of Pharmaceutical Sciences and Technologies (SFSTP) for analyses in biological matrices.[22] Data were treated with the online e.noval™ system (version 1.0) from Arlenda (Liège, Belgium), which is compliant to ICH, FDA, and ISO regulatory documents. Validation criteria were the selectivity, the response functions (calibration curves), the linearity, the precision, the accuracy, the limits of detection, the limits of quantification, and the concentration ranges.

RESULTS AND DISCUSSION

Selectivity

Selectivity was demonstrated by examining potential interferences between plasma components, bromazepam, omeprazole, and oxazepam. To this end, seven different human plasma samples were tested. However, the selectivity of the method with respect to metabolites of bromazepam, i.e., 3-hydroxy-bromazepam and 2-(2-amino-5-bromo-3-hydroxybenzoyl)pyridine[23,24] and of omeprazole, i.e., omeprazole sulfone, hydroxyomeprazole, and omeprazole sulfide,[20,25] could not be ascertained given the non-availability of these compounds.

Figures 4 and 5 compare a typical chromatogram of unspiked plasma with a chromatogram of a plasma sample spiked with bromazepam, omeprazole, and oxazepam. No interference was seen with any of the plasma samples examined.

Response Functions (Calibration Curves) and Linearity

The calibration curve of bromazepam was determined with four calibration standards, covering a range from 5 to 100 ng/mL. The best correlations were linear ones, as reported in Table 1 for the three series of determination

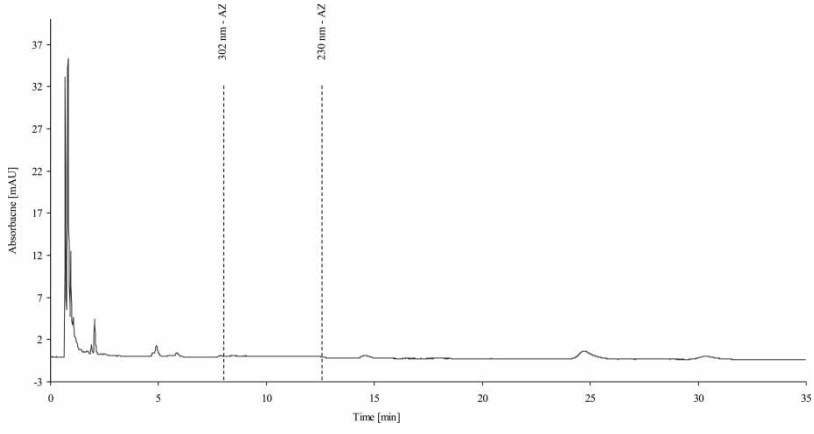

Figure 4. Typical chromatogram of a blank plasma sample obtained by solid-phase extraction, with detection set at 230 nm until 8 min after injection, then at 302 nm until 12.5 min, and finally back at 230 nm. Each vertical dotted line indicates a change in wavelength followed by autozero resetting.

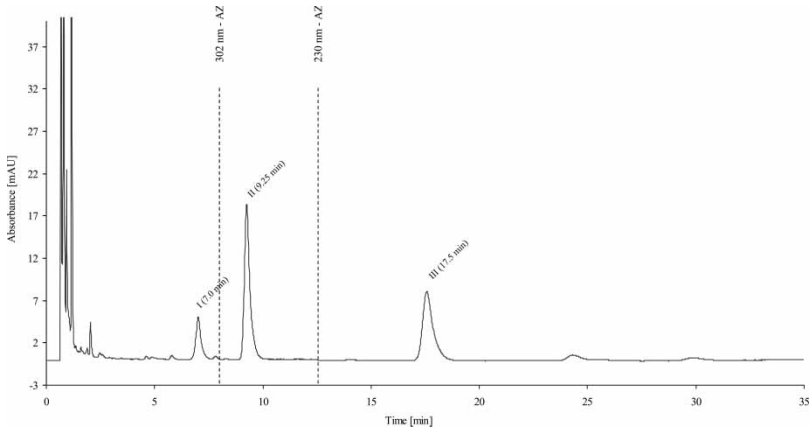

Figure 5. Typical chromatogram of a plasma sample spiked with bromazepam (I: 50 ng/mL), omeprazole (II: 1000 ng/mL) and oxazepam as internal standard (III). Other conditions as in Figure 4.

The calibration curve of omeprazole was determined with five calibration standards, covering a range from 20 to 2000 ng/mL. The best linear correlation was obtained over the full concentration range using weighted linear regression. A weighing factor 1/x allowed heteroscedasticity to be minimized. The statistics of the three series of determination are reported in Table 1.

A linear regression model was determined by correlating the calculated versus nominal concentrations in the three series and over the complete

Table 1. Statistics of the 3 calibration curves determined for bromazepam and omeprazole

Series	Origin	Slope	r^2
Bromazepam (k = 3; n = 3; m = 4)[a]			
1	$-2.84 \cdot 10^{-4}$ ($\pm 2.1 \cdot 10^{-3}$)[b]	$3.20 \cdot 10^{-3}$ ($\pm 3.7 \cdot 10^{-5}$)	0.9981
2	$-2.64 \cdot 10^{-3}$ ($\pm 1.8 \cdot 10^{-3}$)	$3.17 \cdot 10^{-3}$ ($\pm 3.2 \cdot 10^{-5}$)	0.9986
3	$-2.35 \cdot 10^{-3}$ ($\pm 7.7 \cdot 10^{-4}$)	$3.19 \cdot 10^{-3}$ ($\pm 1.4 \cdot 10^{-5}$)	0.9997
Omeprazole (k = 3; n = 3; m = 5)			
1	$1.72 \cdot 10^{-4}$ ($\pm 2.2 \cdot 10^{-3}$)	$1.40 \cdot 10^{-3}$ ($\pm 1.2 \cdot 10^{-6}$)	0.9986
2	$3.08 \cdot 10^{-3}$ ($\pm 1.4 \cdot 10^{-3}$)	$1.33 \cdot 10^{-3}$ ($\pm 7.3 \cdot 10^{-6}$)	0.9996
3	$2.22 \cdot 10^{-3}$ ($\pm 1.2 \cdot 10^{-3}$)	$1.32 \cdot 10^{-3}$ ($\pm 6.3 \cdot 10^{-6}$)	0.9998

[a]k: number of series; n: number of replicates; m: number of conc. levels.
[b]SD in parentheses calculated with Microsoft Excel 2000, Redmond, USA.

concentration ranges. The resulting equations are:

$$\text{for bromazepam: } y = 0.995\,(\pm 0.005) \times -0.21\,(\pm 0.34) \quad (1)$$
$$r^2 = 0.999;\ n = 36$$

$$\text{for omeprazole: } y = 0.996\,(\pm 0.006) \times +6.57\,(\pm 7.06) \quad (2)$$
$$r^2 = 0.998;\ n = 48$$

In Equation (2), the value of the marginally significant origin (6.57) is explained by the fact that the limit of quantification was found to be 20 ng/mL (see below). The corresponding value for bromazepam was 5 ng/mL.

Precision and Trueness

The precision in the quantitative determination of bromazepam and omeprazole was determined by evaluating repeatability (intra-assay precision) and intermediate precision (inter-assay precision). For bromazepam, repeatability was determined by measuring four replicates of three calibration standards (5, 50, and 100 ng/mL). Intermediate precision was evaluated by repeating these measurements three times. For omeprazole, the same procedures were used, but using four calibration standards (20, 40, 1000, and 2000 ng/mL).

The results of this validation step are presented in Table 2, where repeatability and intermediate precision are documented by the standard deviations

Table 2. Repeatability and intermediate precision in the quantitative determination of bromazepam and omeprazole

	Repeatability		Intermediate precision	
Concentration (ng/mL)	SD	RSD (%)	SD	RSD (%)
Bromazepam				
5	0.37	7.3	0.44	8.8
50	0.89	1.8	1.22	2.5
100	1.22	1.2	1.96	2.0
Omeprazole				
20	1.93	9.7	2.87	14.4
40	0.72	1.8	0.74	1.9
1000	15.07	1.5	41.23	4.1
2000	26.08	1.3	66.33	3.3

Table 3. Trueness statistics in the quantitative determination of bromazepam and omeprazole

Nominal concentration (ng/mL)	Calculated concentration (ng/mL)	Absolute error (ng/mL)	Relative error (%)
Bromazepam			
5	4.8	−0.2	−3.7
50	49.4	−0.6	−1.1
100	99.3	−0.7	−0.7
Omeprazole			
20	17.7	−2.4	−11.8
40	43.2	3.2	7.9
1000	993.3	−6.7	−0.7
2000	1929	−70.7	−3.5

(SD) and relative standard deviations (RSD in %) calculated for each nominal concentration.

The accuracy of the method was determined by calculating the absolute errors, the relative errors, and the percentage of overlap of calculated over nominal concentrations (Table 3).

Limits of Detection and Quantification; Concentration Ranges

The limits of detection (LOD) of bromazepam (4.6 ng/mL) and omeprazole (2.0 ng/mL) were estimated based on the mean origins and on the residual variance of the regressions.

The lower and upper limits of quantification (LOQ) of bromazepam are 5 and 100 ng/mL, respectively. These limits define the quantifiable concentration range. Based on these limits, the concentrations of the standards for quality control were set at 5, 50, and 100 ng/mL. At 5 ng/mL, the method shows a 31.4% risk of yielding a result outside the 15% margin of tolerance. This was deemed acceptable since such low levels are not expected in the main part of our pharmacokinetic study. Only in the final elimination phase can such low concentrations be reached, when trends rather than precise concentrations are needed.

For omeprazole, the lower and upper LOQ are 20 and 2000 ng/mL, respectively. The concentrations of the standards for quality control were set at 20, 1000, and 2000 ng/mL. At 20 ng/mL, the method produces a 28.9% risk to obtain a result outside the 20% margin of tolerance. As with bromazepam, this risk was considered acceptable since it is associated with the lower LOQ only.

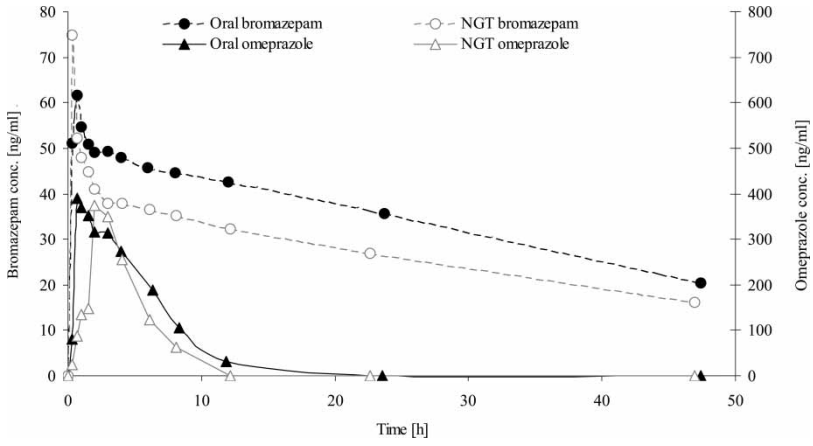

Figure 6. Plasma concentrations determined in a healthy volunteer after simultaneous (interval < 3 min) administration of a single dose of 3 mg of bromazepam (●, ○) and a single dose of 20 mg of omeprazole (▲, △) either orally (●, ▲) or by nasogastric tube (○, △).

Quantitative In Vivo Determination

The quantitative method presented here is now being used in a clinical investigation (accepted by the Ethics Committee) to monitor the plasma concentrations of bromazepam and omeprazole in 8 healthy volunteers. Each volunteer received simultaneously, and on separate occasions either orally and or by nasogastric tube, 3 mg bromazepam plus 20 mg omeprazole, the objective being to ascertain their bioavailability when administered simultaneously by oral or nasogastric route. The wash out period between administrations was 28 days.

The plasma concentration curves for bromazepam and omeprazole from one volunteer are presented in Figure 6. As suggested by these preliminary results, the area under the curve (AUC) of the two drugs appear marginally lower after nasogastric relative to oral administration. This observation suggests a good bioequivalence via nasogastric administration, as well as the absence of any incompatibility with polymeric material. The full results of the study will be published in due time and should clarify this issue.

CONCLUSION

The present method allows the simultaneous HPLC-UV monitoring of bromazepam and omeprazole in human plasma. The limit of quantification of bromazepam achieved here is higher than that reported by Lerner et al.[26] for the sole monitoring of this drug. Furthermore, our LOQ is comparable to that achieved by Tanaka et al.[21] in the simultaneous monitoring of several

benzodiazepines. Our LOQ for omeprazole, although lower than that reported by Motevalian et al.,[20] is higher than most analytical protocols for the drug measured alone.[19,27–30] Nevertheless, the validated concentration range allows the fast, reliable, and simultaneous monitoring of the two drugs administered at usual doses, given a satisfactory specificity, linearity, precision, and trueness. In the clinical investigation now being prepared for publication, the method appears well suited to compare the oral versus nasogastric bioavailabilities of bromazepam and omeprazole administered together or separately.

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