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Highlights:

- A LC/MS/MS method to quantify eight BZDs in breastmilk and plasma was developed.
- The method requires low volume of human breastmilk and plasma (100 μ L).
- LLOQs in breastmilk ranged from 0.25 to 0.5 ng/mL.
- LLOQs in plasma ranged from 0.5 to 1.0 ng/mL.
- The method was successfully applied to characterize alprazolam transfer into milk.

Quantification of eight benzodiazepines in human breastmilk and plasma by liquid-liquid extraction and liquid-chromatography tandem mass spectrometry: Application to evaluation of alprazolam transfer into breastmilk

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Abstract

Breastfeeding is strongly encouraged for infant and maternal health. Benzodiazepines (BZDs) are widely prescribed drugs for symptoms, such as anxiety and insomnia, which many women could experience during the postpartum period. However, limited information is currently available to evaluate the transfer of different BZDs into breastmilk. In order to assess the proprieties of this medication during breastfeeding, robust and sensitive analytical methods to quantify BZDs are required. For this purpose, we developed a method for quantification of BZDs, including alprazolam, bromazepam, clonazepam, clotiazepam, etizolam, flunitrazepam, lorazepam, and CM7116 (a metabolite of ethyl loflazepate), in human breastmilk and plasma using liquid chromatography/tandem mass spectrometry (LC/MS/MS). Sample preparation was performed by a simple liquid-liquid extraction (LLE) with ethyl acetate. For sample preparation of CM7116, the pretreatment process to completely obtain the metabolite was added before the LLE step. The BZDs were separated by a C₁₈ column using a gradient elution of acetonitrile in aqueous ammonium acetate solution, and were detected in the positive ion electrospray mode with multiple reaction monitoring (MRM). Lower limits of quantification (LLOQs) in breastmilk ranged from 0.25 to 0.5 ng/mL, and those in plasma ranged from 0.5 to 1.0 ng/mL. The intra-day and inter-day precision, and accuracy of data were assessed and found to be acceptable. The developed method was successfully applied to measure the concentration of alprazolam in breastmilk and plasma, which were donated by a lactating woman who had been regularly treated with alprazolam. Milk to plasma (M/P) ratios were calculated as 0.52 (before oral administration) and 0.49 (2 h after administration) 3 days after delivery. The M/P ratio 1 month after delivery was calculated as 0.41 (2 h after administration). We estimated that the relative infant dose (RID) values of alprazolam ranged from 3.11 to 4.61%.

Keywords: benzodiazepines, breastmilk, plasma, LC/MS/MS, alprazolam.

Abbreviations: BZD, benzodiazepines; IS, internal standard; LC/MS/MS, liquid chromatography/tandem mass spectrometry; LLE, liquid-liquid extraction; LLOQ, lower limit of quantification; R.E., relative error; RID, relative infant dose; R.S.D., relative standard deviation; MRM, multiple reaction monitoring

1. Introduction

Breastfeeding is highly recommended because of various benefits for the health of both mother and breastfed infant, as well as good nutrition. For example, reduction of the risk of development of ovarian and breast cancers, and diabetes in lactating mothers have been reported [1]. For breastfed infant, positive effects on intelligence quotient and the development of cognitive ability and prevention of various diseases (e.g., infection, diabetes), have been reported [1]. However, breastfeeding in infancy should be tightly controlled if the mother is medicated. Drugs are thought to be transferred into breastmilk to some extent by passive diffusion and carrier-mediated mechanisms [2]. In general, drugs with low ionization, low plasma protein binding, low molecular weight, and high lipophilicity tend to be transferred into breastmilk [2]. Information on the properties of specific drug transfer into breastmilk and safety is important for adequately encouraging women to breastfeed.

Anxiety and insomnia are commonly occurring conditions among pregnant and postpartum women [3]. Untreated maternal anxiety-related illness and abrupt discontinuation of psychotropic drugs could lead to negative effects [4]. Therefore, evaluation of the transfer of psychoactive drugs into breastmilk and the effects of these drugs on breastfed infants are important both for maternal and infant health. Benzodiazepines (BZDs) act as positive allosteric modulators of GABA_A receptor, and are widely prescribed hypnotic, anxiolytic, muscle relaxant, and anticonvulsant drugs. Rubin *et al.* estimated that the adverse event rates of breastfed infants were 17% (1 out of 6), 22% (2 out of 9), and 50% (1 out of 2) when exposed to alprazolam, diazepam, and clonazepam, respectively, after an electronic search study [5]. Kelly *et al.* reported that infant sedation was identified in only 1.6% (2 out of 124) of the infants exposed to BZDs by a telephonic follow-up study [6]. The lack of information on the concentrations of the drugs in breastmilk was considered as one of the major limitations of these studies. BZDs have low molecular weight, high lipophilicity, and high plasma protein binding ratio as their common characteristics [7-9]. However, these characteristics differ to some extent among the different BZDs and the half-life of each BZD also varies [7-9]. Currently, detailed data of transfer of each BZD into breastmilk are limited. Therefore, investigation of the

transfer of BZDs into breastmilk and methods for quantifying these drugs are significant for correct guidance of breastfeeding in woman under medication.

Liquid chromatography/tandem mass spectrometry (LC/MS/MS) is widely used in the quantification of various drugs owing to its high sensitivity, specificity, and capability for simultaneous analyses. Several groups have developed LC/MS/MS methods for the analysis of BZDs in various biological matrix [10], including blood samples (plasma, serum, whole blood) [11-18], urine [17-20], hair [21], and oral fluid [22]. Most of these methods were developed for application in forensic and toxicological sciences. However, only a few methods are currently available for quantifying BZDs in breastmilk samples using LC/MS/MS. Recently, López-García *et al.* reported a method to quantify 40 legal and illegal psychoactive drugs, including several BZDs (alprazolam, diazepam, lorazepam, oxazepam, lormetazepam, temazepam) in breast and bovine milk [23]. Although the group successfully applied the method to quantify caffeine in human breastmilk, the reported method was not validated and was not applied for the quantification of BZDs.

The purpose of this study was to develop a simple and robust analytical method using LC/MS/MS to measure BZDs in human breastmilk and plasma. In the present study, we have chosen the BZDs which are mainly prescribed for the treatment of anxiety. As analytes, we included eight BZDs namely, alprazolam, bromazepam, clonazepam, clotiazepam, etizolam, flunitrazepam, lorazepam, and CM7116 (a metabolite of ethyl loflazepate). The drugs chosen in the present study were prescribed to the breastfeeding women by the Obstetrics Department of Hokkaido University Hospital (unpublished data). Ethyl loflazepate is a drug, which is approved in approximately 10 countries including Japan. The drug is widely used in the Japanese population [24]. After intestinal absorption, the drug is immediately and completely transformed to an unstable metabolite (M-1, CM6913), which is then partially decarboxylated to another metabolite (M-2, CM7116) [25, 26]. It has been reported that ethyl loflazepate is not detected in the plasma after oral administration. Therefore, we developed a quantification method for ethyl loflazepate as a metabolite (CM7116). Using this method, we investigated the levels of alprazolam in human breastmilk (colostrum and

mature milk) and plasma obtained from a patient, and successfully evaluated the transfer of the drug into breastmilk. Furthermore, free concentrations of alprazolam were estimated using the ultrafiltrate samples. This was because BZDs show high plasma protein binding ratio [8, 9], which is an important factor affecting the transport of these drugs into breastmilk.

2. Materials and methods

2.1. Ethics

The study was reviewed and approved by the ethics committees of Hokkaido University Hospital (017-0131).

2.2. Chemicals and reagents

Alprazolam (purity $\geq 98.0\%$), bromazepam (purity $\geq 99.0\%$), clonazepam (purity $\geq 99.0\%$), clotiazepam (purity $\geq 98.0\%$), etizolam (purity $\geq 98.0\%$), flunitrazepam (purity $\geq 99.0\%$), lorazepam (purity $\geq 98.0\%$) and CM7116 (7-Chloro-5-(2-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one)) were purchased from Wako (Tokyo, Japan). Etizolam- d_3 was purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade organic solvents (acetonitrile, ethyl acetate, and methanol) were purchased from Wako. HPLC-grade aqueous ammonium acetate solution (1 M) was obtained from Nacalai Tesque (Kyoto, Japan).

2.3. Preparation for calibration curve and quality control (QC)

Standard stock solutions containing mixtures of alprazolam, bromazepam, clonazepam, clotiazepam, etizolam, flunitrazepam, and lorazepam were prepared in methanol (10, 20, 40, 100, 200, 400, 1000, 2000, 4000, and 10000 ng/mL). CM7116 standard stock solutions were prepared in methanol (10, 20, 40, 100, 200, 400, 1000, 2000, and 4000 ng/mL). An internal standard (IS) stock solution containing etizolam- d_3 (1 $\mu\text{g/mL}$) was also prepared in methanol. All stock solutions were stored at -80°C . The calibration standards and quality control (QC) samples were prepared at the

time of assay by appropriate dilution of the stock solutions in 100 μ L of blank plasma or breastmilk. The concentrations of the calibration standards in breastmilk were 0.25, 0.5, 1, 2.5, 5, 10, and 25 ng/mL for alprazolam; 0.5, 1, 2.5, 5, 10, 25, 50, and 100 ng/mL for bromazepam, clonazepam, etizolam, and lorazepam; and 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, and 100 ng/mL for clotiazepam, flunitrazepam, and CM7116. The concentrations of the calibration standards in plasma were 0.5, 1, 2.5, 5, 10, and 25 ng/mL for alprazolam and lorazepam; 0.5, 1, 2.5, 5, 10, 25, 50, 100, and 250 ng/mL for bromazepam, clonazepam, and flunitrazepam; 0.5, 1, 2.5, 5, 10, 25, 50, and 100 ng/mL for clotiazepam; 0.5, 1, 2.5, 5, 10, 25, and 50 ng/mL for etizolam; and 1, 2.5, 5, 10, 25, 50, and 100 ng/mL for CM7116. The concentrations of the QC samples in breastmilk were 0.25, 0.5, 1, and 10 ng/mL for alprazolam; 0.5, 1, 10, and 100 ng/L for bromazepam, clonazepam, etizolam, and lorazepam; and 0.25, 0.5, 10, and 100 ng/mL for clotiazepam, flunitrazepam, and CM7116. The concentrations of the QC samples in plasma were 0.5, 1, 5, and 25 for alprazolam, etizolam, and lorazepam; 0.5, 1, 25, and 250 for bromazepam, clonazepam, and flunitrazepam; 0.5, 1, 10, and 100 clotiazepam; and 1, 2.5, 50, and 100 ng/mL for CM7116.

2.4. Sample pretreatment

Sample was prepared by liquid–liquid extraction (LLE) method. For quantification of CM7116 (a metabolite of ethyl loflazepate), 50 μ L of 0.5 N HCl was added to 100 μ L of each sample to convert an unstable metabolite CM6913 (M-1) to CM7116 (M-2). The samples for quantification of CM7116 were kept for 30 min at room temperature. Then, the sample was neutralized by adding 25 μ L of 1 N NaOH, and 10 μ L of IS solution was added. After treatment, 10 μ L of IS solution was added to the samples and LLE was performed as described below. For quantification of BZDs expect for CM7116, 10 μ L of IS solution was added to 100 μ L of plasma or breastmilk sample. Subsequently, to each sample, 100 μ L of borate buffer (pH 9, 0.1 M) was added and mixed. For extraction, 1500 μ L of ethyl acetate was added to the sample and vortexed for 10 min. The sample was centrifuged at $3,900 \times g$ for 15 min at 4 $^{\circ}$ C. The upper organic layer was carefully

collected and dried under a nitrogen gas stream at 40 °C. The sample was reconstituted in 100 µL of mobile phase (acetonitrile:10 mM ammonium acetate solution, 30:70, v/v) and filtrated with a DISMIC-13HP filter (0.2 mm, ADVANTEC, Tokyo, Japan). Ten micro liters of sample was injected to LC/MS/MS.

2.5. LC/MS/MS analysis

Chromatographic separation was carried out using a Shimadzu Prominence 20A System (Shimadzu, Kyoto, Japan) and an Inertsustain C18 column (2.0 × 150 mm, 3 µm GL Science Inc., Tokyo, Japan). A binary mobile phase consisted of acetonitrile and 10 mM ammonium acetate (pH 6.8) solution was flown through the apparatus at a rate of 0.2 mL/min. The acetonitrile composition of the mobile phase was increased from 30% to 90% in a linear gradient over 6 min and maintained at 90% for the first 9.0 min. Acetonitrile composition was then decreased to 30% from 9.0 min to 9.5 min and maintained at 30% until 15 min. The column temperature was maintained at 40 °C. The total run time was 15 min. Positive ion electrospray (ESI)-MS/MS analysis was performed using an API 3200™ LC/MS/MS System with multiple reaction monitoring (MRM) (Applied Biosystems, Foster City, CA). MRM was performed by monitoring the transitions summarized in Table 1. Parameter settings were as follows: source temperature of 600°C, spray voltage of 5500 V, curtain gas of 30 psi, ion source gas1 of 40 psi, ion source gas2 of 50 psi, and collision gas of 6 arbitrary units. Data were acquired and analyzed using Analyst software (Applied Biosystems).

2.6. Method validation

The present method was validated in accordance with the guidelines (FDA, Guidance for Industry: Bioanalytical Method Validation (2013) and EMEA, Committee for Medicinal Products for Human Use, Guideline on Bioanalytical Method Validation (2011)). For method validation, individual breastmilk from four normal female donors was purchased from Lee BioSolutions (Maryland Heights, MO). A lot (No. 1) was used for all validation assays, calibration curve,

precision and accuracy, recovery, matrix effect, stability, and carry-over. Three other lots (No. 2 - 4) were used for matrix effect assessment. In addition, blank breastmilk was provided by two healthy volunteers. Pooled plasma from normal human donors was obtained from Cosmo Bio (Tokyo, Japan) and used for method validation including calibration curve, precision and accuracy, recovery, stability, carry-over, and dilution integrity. For the assessment of matrix effect, individual plasma from six normal female donors was purchased from Cosmo Bio (three lots) and Lee BioSolutions (three lots).

2.6.1. Linearity of calibration curves

Calibration curves were constructed using stock solutions in 100 μ L of blank breastmilk or plasma in the ranges listed in Table 3 and Table 4. Calibration curves consisted of at least six concentrations. The samples were pretreated as described in section 2.4 and analyzed. Calibration curves were constructed by plotting the peak area ratio (standard to internal standard) versus the nominal concentration and were fitted using least-squares regression with 1/x weighting.

2.6.2. Precision, accuracy, lower limit of quantification (LLOQ), and recovery

Intra-day precision and accuracy were assessed by analyzing six replicates at four different concentrations on the same day. Inter-day precision and accuracy were assessed by analyzing the replicates at four different concentrations on nine different days. The replicates were prepared and analyzed. The replicates were prepared and analyzed. The R.E. (%) was calculated as [(found concentration – theoretical concentration)/theoretical concentration] $\times$ 100 (%). The precision was obtained as the relative standard deviation (R.S.D.). The acceptable limit for accuracy and precision was $\leq \pm 15\%$ except for LLOQ, for which the acceptable limit was $\leq \pm 20\%$. LLOQ was defined as the concentration with a signal-to-noise (S/N) ratio of at least 10 and precision and accuracy data. Recovery was assessed by spiking known amounts of BZDs into blank plasma or blank breastmilk

and comparing the peak areas of analytes spiked before sample preparation with those of analytes spiked after sample preparation that represent 100% recovery.

2.6.3. Matrix effects

As described above, six plasma samples from healthy female donors were purchased. With regard to breastmilk, two samples from healthy female volunteers and four sample purchased from the seller (Lee BioSolutions) were used for the assay. For each BZD, the matrix effect was assessed by measuring the peak area in the presence of matrix (peak area of the analyte spiked after sample preparation), and the peak area in the absence of matrix (peak area of an equivalent amount of analyte prepared in the mobile phase). The matrix effect was calculated using the following equation;

$$\text{Matrix effect (\%)} = \left(\frac{\text{Peak area in the presence of matrix}}{\text{Peak area in the absence of matrix}} \times 100 \right) - 100$$

Furthermore, the accuracy and precision in multiple lots were assessed. For assessment in breastmilk, known amounts of BZDs were spiked in four lots of breastmilk purchased from Lee BioSolutions (Lot.1 - 4). The samples were prepared as described in Section 2.4, then analyzed. The concentration of the analytes in each lot was quantified using a calibration curve prepared for Lot.2. For assessment of plasma, three lots of plasma obtained from Cosmo Bio (Lot.1 - 3) were assessed. The calibration curves were prepared in pooled plasma

2.6.4. Stability

The stability of BZDs in breastmilk and plasma was investigated. The short-term stability was assessed after storing BZDs for 24 h in breastmilk or plasma at 4 °C. The long-term stability was assessed after storing BZDs for 8 weeks in breastmilk or plasma at – 30 °C. Freeze-thaw stability was assessed after three freeze-thaw cycles (–30°C to room temperature).

2.6.5. Carry-over

The carry-over was assessed by injection of a blank sample after injection of the highest calibration sample. The area responses of the blank samples were compared to the mean area response of the LLOQ. The peak area of the blank samples following the highest calibration should not exceed 20% of the peak area of the LLOQ and 5% of the peak area of the IS.

2.6.7. Dilution integrity

The dilution integrity was assessed with the plasma samples. The analytes in the plasma at a concentration of 250 ng/mL were diluted 10-fold with blank plasma. Six replicates were analyzed, and the accuracy and precision were calculated. According to the EMA guideline, the precision and accuracy should not exceed $\pm 15\%$.

2.7. Application to clinical samples

Samples for quantification of BZD in breastmilk and plasma were obtained from a lactating woman who was regularly administrated with alprazolam at Hokkaido University Hospital. The volunteer gave written, informed consent, which was approved by the institutional review board. The volunteer participated in the study three days after the delivery and one month after postnatal checkup. Samples were collected before and 2 h after oral administration of alprazolam. A dose of 0.8 mg/day was administrated for 3 days after delivery, and 1.0 mg/day for 1 month after the delivery. The breastmilk and plasma samples were stored at $-30\text{ }^{\circ}\text{C}$ until analysis. For assessment of protein binding of alprazolam, we obtained ultrafiltrates of plasma and breastmilk using Centrifree® Ultrafiltration Devices (Merck Millipore, Tulagreen, Ireland) in accordance with the manufacturer's instruction. For quantification of alprazolam in ultrafiltrate samples, calibration solutions were prepared in 100 μL of ultrafiltrate blank plasma or ultrafiltrate blank breastmilk.

M/P ratios were calculated as the total concentration in breastmilk/total concentration in plasma. RID was estimated as:

$$\text{RID (\%)} = \frac{\text{Total concentration in breastmilk (mg/mL)} \times \text{Infant intake of breastmilk (mL/kg/day)}}{\text{Maternal intake dose (mg/kg/day)}} \times 100$$

where “Infant intake of breastmilk (mL/kg/day)” was used the average value “150 (mL/kg/day)”.

Protein binding ratio was calculated as [(total drug concentration – free drug concentration)/total drug concentration] × 100 (%).

3. Results and discussion

3.1. LC/MS/MS

In the present study, ESI-MS/MS (positive mode) was used for detecting the BZDs: alprazolam, bromazepam, clonazepam, clotiazepam, etizolam, flunitrazepam, lorazepam, and CM7116 (a metabolite of ethyl loflazepate). Positive ion mass spectra indicated the presence of the protonated molecules for each compound (data not shown). Product ions with high intensity were selected for analysis. Since bromazepam has a bromine in the structure, two intense ions (m/z 316 and m/z 318) were observed. Although the intensity of m/z 316 > 182 was higher than that of m/z 318 > 182, the interfering peak was observed in blank breastmilk when the transition m/z 316 > 182 was monitored. Therefore, m/z 318 was selected as a precursor ion and the transition m/z 318 > 182 was monitored for the detection of bromazepam. Table 1 shows the ion pairs selected for MRM and parameter settings. Many studies have monitored multiple transitions with each analyte, whereas several studies have also used a single transition for quantification of BZDs [11, 16]. Monitoring multiple transitions for each analyte is advisable to increase the specificity and reduce the risk of false positives. Although we selected a single transition for quantification of each analyte, no interfering peak was observed in any of the tested lots of blank plasma and blank breastmilk samples. The transitions, as shown in Table 1 were monitored.

Various analytical columns, including C18 [11, 13-18, 20-22] and phenyl types [12, 19], have been applied for the separation of BZDs. In our preliminary study, two types of columns (C18 column or phenyl-hexyl column) were tested. Separation pattern of the analytes between these columns was not largely different. In the present study, a C18 column (Inertsustain C18, 2.0×150 mm, $3 \mu\text{m}$) was used for simultaneous analysis. Furthermore, several mobile phases, different combinations of organic solvents (methanol and acetonitrile) and aqueous solutions (10 mM ammonium formate plus 0.1% formic acid, 10 mM ammonium acetate, 10 mM ammonium bicarbonate), were tested. When the solution having a lower pH (10 mM ammonium formate plus 0.1% formic acid) was tested, the sensitivity of the analytes (bromazepam and lorazepam) was lower

than in solutions having a higher pH [10 mM ammonium acetate (pH 6.8) or 10 mM ammonium bicarbonate (pH 8)]. Since sharp peaks were obtained with ammonium acetate rather than with ammonium bicarbonate, ammonium acetate solution was selected for this study. Although both methanol and acetonitrile produced sharp peaks with optimal separation and sensitivity, acetonitrile was selected as an organic solvent because it created lower pressure on the column. Therefore, a gradient elution with 10 mM ammonium acetate and acetonitrile as described in 2.5, was selected for this study. The total run time was 15 min including column equilibration. Figure 1 shows the representative chromatograms of the blank sample (A and C), the blank sample with LLOQ levels of BZDs (B and D). As shown in the figures, no significant interference was observed in the blank sample at the time of retention of each BZD, showing that the method has optimal specificity.

3.2. Sample preparation

Several developed methods quantify BZDs in plasma, including ethyl loflazepate, as a parent drug [11, 17]. As mentioned in the *Introduction* section, ethyl loflazepate is immediately transformed to an unstable carboxylic metabolite (M-1, CM6913), which is then partially decarboxylated to another metabolite (M-2, CM7116) [25, 26]. Sample treatment for completely changing to CM7116 was based on the previous reported method with some modifications [25]. As described in 2.4, plasma and breastmilk sample for quantifying CM7116 was first treated with HCl and then neutralized with NaOH before LLE.

Several methods for sample preparation of BZDs, including LLE [12-15, 22], solid-phase extraction [11, 16, 17], protein precipitation [20], have been reported. In the present study, a simple LLE method was applied for the quantification of the eight BZDs. Ethyl acetate was the organic solvent used in the extraction. The recovery from breastmilk ranged from 56.5 to 83.8% and that from plasma ranged from 66.6 to 116.7% (Table 2). Compared to the previously described methods quantifying BZDs such as alprazolam bromazepam, clonazepam, flunitrazepam, and lorazepam in

plasma [13-15, 17, 18], a smaller sample volume was needed for the present method (100 μ L) to have good sensitivity. In their method, Simonsen *et al.* [15] and Verplaetse *et al.* [17] have reported the LLOQs of alprazolam, bromazepam, clonazepam, flunitrazepam, and lorazepam as 2-5 ng/mL. Marin *et al.* [14] have reported the LLOQs of the same BZDs were 1 ng/mL, whereas Montenarh *et al.* [13] have reported that LLOQs of the same BZDs were 5–40 ng/mL. The LLOQs of alprazolam, clonazepam, flunitrazepam, and lorazepam were found to be 1–5 ng/mL in the method by Mata *et al.* [18]. In the present study, the LLOQ of the same BZDs in plasma was 0.5 ng/mL. The previously reported methods required larger sample volumes (200–500 μ L) than the present method. López-García *et al.* used protein precipitation by methanol for quantification of psychoactive drugs, such as alprazolam and lorazepam, in breastmilk [23]. The LLOQs of alprazolam and lorazepam were 0.5 ng/mL and 3.0 ng/mL, respectively. The method used 0.5 mL of breastmilk. A smaller sample volume was needed to carry out the present method compared to the method [23].

3.3. Method validation

3.3.1. Calibration curve

Calibration standards were constructed by spiking at least six different concentrations of analytes to blank breastmilk or plasma. In the ranges showed in the Table 3 and Table 4, the present method presented good linearity both in breastmilk ($r^2 > 0.997$) and in plasma ($r^2 > 0.993$).

3.3.2. Accuracy and precision

The intra-day precision and inter-day precision as well as accuracy were tested at four different concentrations. The data are summarized in Table 3 (breastmilk) and Table 4 (plasma). In the quantification method with breastmilk, the intra-day precision ranged from 1.3 to 17.7% and the accuracy ranged from – 19.4 to 18.2%. The inter-day precision ranged from 2.5 to 13.2% and the accuracy ranged from – 20.0 to 10.1%. In the quantification method with plasma, the intra-day precision ranged from 2.2 to 12.1% and the accuracy ranged from – 19.2 to 16.6%. The inter-day

precision ranged from 1.8 to 14.4% and the accuracy ranged from – 14.3 to 16.2%. The precision and accuracy were within 15%, except for LLOQ (those of LLOQ were within 20%). These results indicate that the present method is highly reliable and has good accuracy and precision.

3.3.3. Matrix effect

The results of the matrix effect assessment are summarized in Table 5. Ion suppression was observed with most of the analytes, especially clonazepam in both breastmilk and plasma. In the present study, we used deuterium-labeled etizolam as the internal standard for all analytes. Therefore, we investigated the accuracy and precision in multiple lots of breastmilk and plasma to assess whether the IS can compensate for variations in matrix effect and recovery. As shown in Table 6, no significant variability among the lots was observed. During quantification of breastmilk, the precision among the lots ranged from 2.2 to 19.6% and the accuracy ranged from – 7.4 to 20.5%. With plasma, the precision among the lots ranged from 0.4 to 19.7% and the accuracy ranged from – 11.8 to 17.9%. A single IS for multiple analytes was used in some studies for quantifying BZDs in plasma and urine [11, 12, 16, 17, 21, 22]. In the present method, a single IS was used as a surrogate IS because of the chemical similarity with BZDs. Considering the validation data (Table 3, 4, and 6) and clinical concentration of BZDs [27], we concluded that the use of a single IS was adequate to quantify BZDs in the present study. The use of an isotopically stable IS for each analyte is, however, generally recommended for LC/MS/MS analysis. In some conditions, for e.g. for detection of a concentration of 1 ng/mL of alprazolam in breast milk, the accuracy and precision were large to some extent. Future studies are needed to assess whether the use of multiple ISs can lead to improvement of accuracy and precision among the lots of matrices.

3.3.4. Stability

We examined the short-term stability of BZDs (for 24 h at 4 °C), long-term stability (for 8 weeks at -30 °C), and freeze-thaw stability both in breastmilk and plasma. The data are summarized in Table 7. After storing BZDs in these conditions, the remaining amounts of the compounds were

quantified. For short-term stability, the remaining amounts of BZDs in the samples ranged from 94.7% to 111.3% in breastmilk, and from 86.0% to 104.0% in plasma. For long-term stability, the remaining amounts of BZDs ranged from 91.2% to 112.5% in breastmilk, and from 92.9% to 118.7% in plasma. After three freeze-thaw cycles, the remaining amounts of BZDs ranged from 82.5% to 105.6% in breastmilk, and from 92.7% to 104.4% in plasma. These results indicated that no significant degradation was observed at least in these conditions.

3.3.5. Carry-over

The carry-over with all the analytes were within 20% of the LLOQ (range: 0 to 9.7% for breastmilk and 0.4 to 9.7% for plasma). The carry-over with IS was < 0.1%.

3.3.6. Dilution integrity

The plasma concentration of different analytes (alprazolam, clonazepam, lorazepam, and CM7116) in an authentic sample may exceed the calibration range based on the reported clinical range [27], whereas the breastmilk concentration of the samples is expected to be within the calibration range. Therefore, we assessed the dilution integrity in the plasma sample. As shown in Table 8, accuracy ranged from – 1.9 to 6.0% and the precision ranged from 2.1 to 6.3%. The results indicated that the plasma concentration of all the analytes up to a concentration of 250 ng/mL could be accurately quantified with 10-fold dilution.

3.4. Application of the method for the assessment of alprazolam transfer into breastmilk

To investigate the suitability of the developed method, the method was applied to quantify alprazolam in breastmilk and plasma samples, which were donated by a lactating woman who had been regularly treated with alprazolam before pregnancy. Samples were collected before (trough) and 2 h after (estimated the time of maximum concentration in plasma) oral administration of alprazolam. Figure 2 shows the chromatograms of the authentic samples. As shown in Figure 2A and 2B, alprazolam was detected both in breastmilk and in plasma samples. No peak other than that of

alprazolam (m/z 309 > 281) was observed. The transitions, as shown in Table 1 were monitored (data not shown). The analyzed data are summarized in Table 9. In the present study, the M/P ratio was calculated to be 0.41 one month after delivery. At 3 days after delivery, M/P ratios were calculated to be 0.52 (trough) and 0.49 (peak). It has been reported that the time profiles of alprazolam concentrations in breastmilk and plasma were in a parallel fashion [28]; the study group reported that the M/P ratio of alprazolam from lactating volunteers 6–28 weeks after single administration of 0.5 mg alprazolam was estimated to be 0.36 ± 0.11 . These results were not significantly different from the results of previous studies, indicating the applicability of the present method for the evaluation of transfer of BZDs into breastmilk.

Three days after delivery, M/P ratios tended to be higher than that after one month. It has been reported that the composition of colostrum varies when it changes to mature milk [29]. Furthermore, it is generally thought that drugs penetrate more in colostrum than in mature milk, because of the incompleteness of tight junctions of mammary gland cells [2]. The differences between colostrum and mature milk may affect the M/P ratio. Since there is limited information about the change over time during lactation, further studies are required to better understand the properties of BZD transfer into breastmilk.

Furthermore, we estimated the RID values from the concentration of alprazolam in breastmilk, the results ranged from 3.11 to 4.61%. Generally, if a RID value of a drug is lower than 10%, it is considered to be compatible with breastfeeding [2]; nevertheless, each one of the individual situations should be taken in account. The present finding may support the propriety of breastfeeding during alprazolam administration. Several studies have reported adverse effects on breastfed infants, such as sedation, although the concentration levels have not been investigated [5, 6]. As per Medications and Mothers' Milk 2017 [2], alprazolam is categorized as L3 (Limited Data-Probably Compatible). In the Drugs in Pregnancy and Lactation (11th edition), the drug is categorized as "Limited Human Data-Potential Toxicity" [30]. Future studies are urgently needed to clarify whether the potential adverse effects are related with concentration levels of BZDs.

As described in the *Introduction* part, BZDs showed high plasma protein binding ratio [8, 9], which is an important factor affecting its transport into breastmilk. Therefore, we quantified ultrafiltrate samples to investigate the concentration of free alprazolam and to estimate the protein binding ratio. As shown in Figure 2C and 2D, alprazolam was detected both in ultrafiltrate breastmilk and ultrafiltrate plasma samples. The percentages of protein binding in plasma ranged from 83.8 to 85.1% and those in breastmilk from 39.5 to 48.8%. The data of plasma protein binding was close to the previous reported values [8]. The results indicated that the present method could be applied for the evaluation of protein binding of BZDs in breastmilk and plasma.

4. Conclusion

In the present study, we developed a method for the quantification of eight BZDs, including alprazolam, bromazepam, clonazepam, clotiazepam, etizolam, flunitrazepam, lorazepam and CM7116 (a metabolite of ethyl loflazepate), in human breastmilk and human plasma, using LC/MS/MS. In the present method, only 100 μ L of breastmilk and plasma were used. Sample preparation was conducted by a simple LLE. For quantification of CM7116, pretreatment process for completely changing to the metabolite was added. Currently, there are few LC/MS/MS methods for the quantification of BZDs in breastmilk. To the best of our knowledge, there are no reports on the determination methods for bromazepam, clonazepam, clotiazepam, etizolam, flunitrazepam, and CM711 in breastmilk sample. Furthermore, the quantification methods for etizolam, clotiazepam, and CM7116 in plasma are also limited compared to other BZDs. The developed method was successfully applied to the measurement of alprazolam in authentic samples. We revealed the concentrations of alprazolam in breastmilk and plasma, which were obtained from a lactating woman who was regularly administered alprazolam. Since there is limited information obtained from patients regularly administered BZD, the findings of the present study may help to identify a better

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therapeutic strategy during breastfeeding. The method described here could be useful for future studies evaluating the properties of BZDs transfer into breastmilk.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Figure captions

Figure 1. Representative chromatograms of analytes in blank human breastmilk and plasma. (A)

Blank breastmilk. (B) Blank breastmilk with LLOQ levels of analytes, (C) Blank plasma, (D) Blank plasma with LLOQ levels of analytes.

Figure 2. Multiple reaction monitoring chromatograms of alprazolam for authentic samples obtained from a patient treated with alprazolam at trough. Chromatograms of (A) breastmilk sample, (B) plasma sample, (C) breastmilk sample ultrafiltrate, and (D) plasma sample ultrafiltrate.

Table 1. MRM parameters for determination of BZDs

Analyte	Precursor ion (<i>m/z</i>)	Product ion (<i>m/z</i>)	Dwell time (msec)	DP (V)	EP (V)	CE (V)	CEP (V)	CXP (V)
Alprazolam	309	281	110	61	8	41	16	8
Bromazepam	318	182	110	56	5.5	45	16	4
Clonazepam	316	214	110	56	4	47	22	4
Clotiazepam	319	154	110	61	6	37	22	4
Etizolam	343	314	110	61	12	29	16	4
Flunitrazepam	314	268	110	66	4.5	27	28	4
Lorazepam	321	275	110	51	5	27	16	4
CM7116	289	140	110	66	4.5	39	14	4
Etizolam-d ₃ (IS)	346	317	110	61	12	29	16	4

DP, Declustering potential; EP, Entrance potential; CE, Collision energy; CEP, Collision cell entrance potential; CXP, Collision cell exit potential.

Table 2. Recovery of BZDs from breastmilk and plasma

		Recovery (%) (Mean $\pm$ S.D., n=3)						
Concentration (ng/mL)		Breastmilk			Plasma			
Alprazolam	0.5	70.6	$\pm$	1.9	77.9	$\pm$	7.0	
	25	71.7	$\pm$	1.1	86.0	$\pm$	5.1	
Bromazepam	0.5	83.8	$\pm$	3.8	73.0	$\pm$	10.6	
	25	66.8	$\pm$	1.7	77.9	$\pm$	8.3	
	100	68.5	$\pm$	2.5	71.7	$\pm$	2.1	
Clonazepam	0.5	77.6	$\pm$	7.6	79.2	$\pm$	14.5	
	25	73.9	$\pm$	1.2	79.2	$\pm$	6.0	
	100	72.7	$\pm$	3.6	72.9	$\pm$	3.9	
Clotiazepam	0.5	57.5	$\pm$	5.4	116.7	$\pm$	8.9	

	25	61.7	±	2.7	85.5	±	6.8
	100	70.6	±	5.2	73.2	±	6.0
	0.5	60.9	±	1.7	92.0	±	3.4
Etizolam	25	68.5	±	2.1	86.1	±	2.9
	100	77.6	±	3.6	-		
	0.5	79.4	±	1.1	76.8	±	4.3
Flunitrazepam	25	72.3	±	2.1	82.5	±	6.0
	100	75.2	±	3.0	73.2	±	3.5
	0.5	76.6	±	7.1	78.9	±	1.7
Lorazepam	25	75.0	±	2.1	84.6	±	8.0
	100	76.6	±	3.9	-		
	0.5	59.6	±	8.4	-		
CM7116	25	56.5	±	7.8	72.8	±	3.5
	100	61.1	±	5.0	66.6	±	6.0

Table 3. Intra-day and inter-day reproducibility of BZDs in breastmilk

	Calibration range (ng/mL)	r^2 (n=6)	Spiked (ng/mL)	Intra-day (n=6)			Inter-day (n=9)		
				Found (ng/mL)	R.S.D. (%)	R.E. (%)	Found (ng/mL)	R.S.D. (%)	R.E. (%)
Alprazolam	0.25 - 25	0.997	0.25	0.221	3.6	-11.7	0.212	13.1	-15.3
			0.5	0.514	4.4	2.7	0.500	5.7	0.1

				1	1.08	4.0	8.2	1.06	7.0	5.7
				10	9.99	1.8	-0.1	10.1	5.8	1.4
Bromazepam	0.5 - 100	0.997	0.5	0.542	7.7	8.4	0.546	8.0	9.3	
			1	1.06	5.9	5.7	1.03	6.7	3.2	
			10	9.85	2.7	-1.5	9.57	8.5	-4.3	
			100	101	2.7	1.0	101	2.7	1.0	
Clonazepam	0.5 - 100	0.998	0.5	0.591	17.6	18.2	0.551	12.5	10.1	
			1	1.14	14.4	14.3	0.923	12.3	-7.7	
			10	9.50	5.6	-5.0	9.74	7.6	-2.6	
			100	101	4.3	0.7	100	3.2	0.5	
Clotiazepam	0.25 - 100	0.998	0.25	0.260	17.7	3.9	0.243	13.1	-2.6	
			0.5	0.551	7.9	10.2	0.482	8.3	-3.6	
			10	9.07	1.5	-9.4	10.1	6.6	1.5	
			100	94.8	5.1	-5.3	99.0	4.0	-1.0	
Etizolam	0.5 - 100	0.998	0.5	0.421	5.0	-15.8	0.437	5.0	-12.6	
			1	0.97	5.3	-3.2	0.956	8.0	-4.4	
			10	10.7	2.9	6.8	10.4	6.5	4.4	
			100	98.1	3.0	-2.0	97.6	3.2	-2.4	
Flunitrazepam	0.25 - 100	0.998	0.25	0.267	7.2	6.6	0.271	12.0	8.2	
			0.5	0.508	8.4	1.6	0.485	10.9	-3.0	
			10	9.58	2.7	-4.2	9.61	7.3	-3.9	
			100	103	3.2	2.6	101	2.9	1.2	
Lorazepam	0.5 - 100	0.997	0.5	0.403	8.2	-19.4	0.400	10.5	-20.0	
			1	1.05	10.8	4.7	0.927	13.2	-7.3	
			10	10.4	1.3	3.8	10.8	5.4	7.9	
			100	97.1	2.9	-2.9	96.4	2.6	-3.6	
CM7116	0.25 - 100	0.998	0.25	0.246	12.3	-1.5	0.226	12.2	-9.6	
			0.5	0.498	3.8	-0.4	0.489	7.5	-2.2	
			10	10.6	4.8	5.6	10.5	3.0	4.7	
			100	94.3	2.4	-5.7	98.4	2.5	-1.6	

Table 4. Intra-day and inter-day reproducibility of BZDs in plasma

	Calibration range (ng/mL)	r^2 (n=6)	Spiked (ng/mL)	Intra-day (n=6)			Inter-day (n=9)		
				Found	R.S.D.	R.E.	Found	R.S.D.	R.E.
				(ng/mL)	(%)	(%)	(ng/mL)	(%)	(%)
			0.5	0.404	5.8	-19.2	0.429	11.3	-14.3
Alprazolam	0.5 - 25	0.993	1	0.926	4.0	-7.4	0.944	5.4	-5.6
			5	5.23	2.3	4.5	5.47	4.7	9.4

			25	22.8	6.6	-8.9	23.9	2.7	-4.2
			0.5	0.544	7.8	8.9	0.581	14.4	16.2
			1	1.04	5.4	3.5	0.919	5.6	-8.1
Bromazepam	0.5 - 250	0.997	25	23.9	7.9	-4.5	24.4	6.2	-2.2
			250	245	2.5	-2.1	251	3.2	0.4
			0.5	0.583	12.1	16.6	0.538	7.7	7.6
			1	0.979	10.5	-2.1	0.977	11.7	-2.3
Clonazepam	0.5 - 250	0.998	25	22.0	9.7	-12.1	24.6	5.3	-1.6
			250	242	3.4	-3.3	253	2.8	1.4
			0.5	0.496	10.7	-0.8	0.457	9.7	-8.6
			1	0.974	3.9	-2.6	1.07	8.4	7.3
Clotiazepam	0.5 - 100	0.999	10	9.30	2.4	-7.0	9.81	3.7	-1.9
			100	92.1	3.4	-7.9	100	1.8	0.5
			0.5	0.410	9.3	-18.0	0.476	10.0	-4.9
			1	0.887	5.1	-11.3	0.972	6.6	-2.8
Etizolam	0.5 - 50	0.999	5	4.97	2.2	-0.6	5.20	4.1	4.0
			25	23.8	6.7	-4.8	25.3	4.2	1.3
			0.5	0.493	7.5	-1.4	0.506	11.8	1.2
			1	0.979	2.4	-2.1	0.96	7.7	-3.8
Flunitrazepam	0.5 - 250	0.998	25	22.6	8.1	-9.6	24.8	5.0	-0.9
			250	238	3.1	-4.8	249	2.7	-0.4
			0.5	0.406	11.4	-18.9	0.441	13.0	-11.8
			1	0.959	5.8	-4.2	0.908	5.0	-9.2
Lorazepam	0.5 - 25	0.993	5	5.24	3.2	4.8	5.44	5.3	8.8
			25	22.1	7.7	-11.7	23.8	2.8	-4.7
			1	0.852	9.3	-14.8	0.900	9.6	-10.0
			2.5	2.47	4.1	-1.3	2.54	4.8	1.7
CM7116	1 - 100	0.999	50	50.6	2.5	1.2	50.5	3.5	0.9
			100	96.1	2.2	-3.9	98.9	2.2	-1.1

Table 5. Matrix effect of BZDs in breastmilk and plasma

	Breastmilk (n=6)		Plasma (n=6)	
	Concentration	Matrix effect	Concentration	Matrix effect
	(ng/mL)	(%)	(ng/mL)	(%)
Alprazolam	0.5	-25.4	1	-5.4
	25	-21.7	25	-10.8
Bromazepam	0.5	-6.5	1	-17.8
	100	-20.6	100	-11.7
Clonazepam	0.5	-27.4	1	-22.0

	100	-18.3	100	-11.1
Clotiazepam	0.5	-45.7	1	-38.1
	100	-52.9	100	-41.6
Etizolam	0.5	-10.0	1	3.2
	100	-17.4	25	-11.9
Flunitrazepam	0.5	-9.0	1	-13.4
	100	-16.8	100	-16.9
Lorazepam	0.5	-29.3	1	-18.2
	100	-14.8	25	-16.6
	0.5	-30.5	1	-17.5
CM7116	100	-21.5	100	-10.8

Table 6. Accuracy and precision in multiple lots

Breastmilk								Plasma					
	Spiked	Found (ng/mL)				R.S.D.	R.E.	Spiked	Found (ng/mL)			R.S.D.	R.E.
		Lot. 1	Lot. 2	Lot. 3	Lot. 4				Lot. 1	Lot. 2	Lot. 3		
	(ng/mL)					(%)	(%)	(ng/mL)				(%)	(%)
Alprazolam	0.5	0.605	0.461	0.573	0.578	11.5	10.9	1	1.01	0.947	1.10	7.5	1.9
	1	1.25	1.10	1.12	1.35	9.7	20.5	5	5.14	5.54	5.26	3.9	6.3
	10	11.9	11.0	9.25	11.5	10.7	9.1	25	23.0	24.3	24.7	3.7	-4.0
Bromazepam	1	1.17	1.08	1.05	1.05	5.2	8.7	1	1.25	1.00	0.903	17.0	5.1
	10	10.9	10.3	9.81	10.3	4.3	3.3	5	4.72	5.32	4.73	7.0	-1.5
	100	124	105	112	127	8.8	17.0	100	96.5	97.9	101	2.3	-1.5
Clonazepam	1	0.862	1.27	0.849	1.03	19.6	0.3	1	1.01	1.26	1.01	13.2	9.3
	10	9.26	9.75	10.4	9.73	4.8	-2.2	5	4.44	5.01	3.78	14.0	-11.8
	100	111	95.5	106	103	6.2	3.9	100	89.8	91.4	88.3	1.7	-10.2
Clotiazepam	0.5	0.556	0.496	0.649	0.542	11.4	12.2	1	1.41	1.18	0.946	19.7	17.9
	10	11.6	10.5	11.1	9.03	10.5	5.6	5	6.40	6.11	4.78	15.0	15.3
	100	113	98.4	119	99.9	9.3	7.6	100	117	118	106	5.9	13.7
Etizolam	1	1.04	1.02	0.998	1.05	2.2	2.7	1	0.961	1.05	0.975	4.8	-0.5
	10	11.2	10.5	10.4	10.1	4.4	5.5	5	5.01	5.11	4.80	3.2	-0.5
	100	98.6	89.8	95.2	98.1	4.2	-4.6	25	23.7	23.8	23.9	0.4	-4.8
Flunitrazepam	0.5	0.471	0.434	0.488	0.497	5.9	-5.5	1	0.900	0.998	1.12	11.0	0.6
	10	10.1	9.52	9.85	9.63	2.6	-2.3	5	4.51	5.03	4.50	6.5	-6.4
	100	115	97.5	103	110	7.2	6.4	100	91.0	99.4	92.3	4.8	-5.8
Lorazepam	1	0.768	0.877	1.03	1.03	13.8	-7.4	1	1.10	0.944	1.10	8.6	4.8
	10	11.4	10.2	9.77	10.9	6.9	5.7	5	5.97	6.01	5.60	3.9	17.2
	100	107	91.9	98.3	104	6.6	0.3	25	24.5	25.1	25.4	1.8	0.0
CM7116	0.5	0.513	0.503	0.394	0.545	13.4	-2.3	2.5	2.37	2.51	2.60	4.6	-0.3
	10	9.61	10.5	10.9	10.1	5.4	2.8	25	24.3	24.8	25.2	1.8	-0.9
	100	108	98.8	100	99.4	4.3	1.6	100	108	101	104	3.4	4.3

Table 7. Stability of BZDs in breastmilk and plasma

	Stability (% remaining) (Mean ± S.D., n=3)															
	Breastmilk									Plasma						
	Concentration (ng/mL)	24 h (4 °C)	8 weeks (-30 °C)	Freeze-thaw (-30°C and room temperature, 3 cycles)	Concentration (ng/mL)	24 h (4 °C)	8 weeks (-30 °C)	Freeze-thaw (-30°C and room temperature, 3 cycles)								
Alprazolam	1	111.3 ± 3.8	96.5 ± 4.3	99.4 ± 3.8	1	89.4 ± 4.7	114.7 ± 3.1	92.7 ± 6.8								
	25	96.3 ± 6.9	96.4 ± 3.3	86.9 ± 2.8	25	93.1 ± 5.4	92.9 ± 1.7	93.7 ± 5.0								
Bromazepam	25	103.2 ± 8.6	91.2 ± 3.0	91.9 ± 4.7	25	100.0 ± 4.9	106.0 ± 1.2	104.4 ± 3.8								
	100	108.3 ± 4.6	96.3 ± 2.7	90.4 ± 2.8	100	96.3 ± 3.5	106.3 ± 4.6	102.5 ± 9.9								
Clonazepam	25	100.3 ± 4.8	99.6 ± 3.8	92.4 ± 1.2	25	94.0 ± 8.3	100.0 ± 3.2	99.3 ± 4.3								
	100	105.7 ± 4.6	104.0 ± 2.6	96.8 ± 1.3	100	94.5 ± 4.1	106.3 ± 4.2	96.6 ± 9.4								
Clotiazepam	25	105.9 ± 9.6	107.7 ± 3.0	82.5 ± 3.8	25	101.3 ± 7.9	98.0 ± 3.6	102.9 ± 4.1								
	100	105.7 ± 4.9	95.5 ± 7.3	91.5 ± 3.0	100	101.1 ± 6.1	108.0 ± 2.6	101.8 ± 8.2								
Etizolam	25	101.5 ± 7.2	102.0 ± 1.4	105.6 ± 3.2	1	86.0 ± 1.4	96.2 ± 9.6	95.4 ± 6.3								
	100	98.4 ± 2.0	95.9 ± 2.6	96.6 ± 1.2	25	91.7 ± 3.4	96.1 ± 2.4	100.0 ± 5.4								
Flunitrazepam	25	95.9 ± 6.5	101.5 ± 0.8	96.0 ± 5.2	25	99.7 ± 5.7	98.8 ± 1.8	99.7 ± 5.4								
	100	105.0 ± 2.6	102.3 ± 1.5	100.0 ± 1.8	100	94.9 ± 4.9	106.0 ± 3.6	98.7 ± 6.8								
Lorazepam	25	100.5 ± 6.1	106.1 ± 4.2	99.6 ± 3.4	1	87.7 ± 9.4	113.7 ± 6.0	99.3 ± 9.0								
	100	95.5 ± 1.6	96.0 ± 1.8	93.1 ± 2.2	25	95.2 ± 8.7	100.5 ± 2.2	92.9 ± 5.8								
CM7116	25	95.9 ± 1.7	112.5 ± 4.6	102.7 ± 7.7	25	104.0 ± 3.3	118.7 ± 1.7	99.6 ± 5.9								
	100	94.7 ± 1.5	99.4 ± 3.7	94.5 ± 6.0	100	90.1 ± 8.6	112.0 ± 3.6	94.8 ± 0.6								

Table 8. Dilution integrity of BZDs in plasma.

	Nominal concentration (ng/mL)	Found concentration (ng/mL)	R.S.D. (%)	R.E. (%)
Alprazolam	25	24.5	3.9	-1.9
Bromazepam	25	26.2	5.6	4.7
Clonazepam	25	25.2	6.3	0.7
Clotiazepam	25	26.4	2.1	5.7
Etizolam	25	25.4	4.9	1.5
Flunitrazepam	25	26.5	3.3	6.0
Lorazepam	25	26.3	4.6	5.1
CM7116	25	24.9	4.0	-0.4

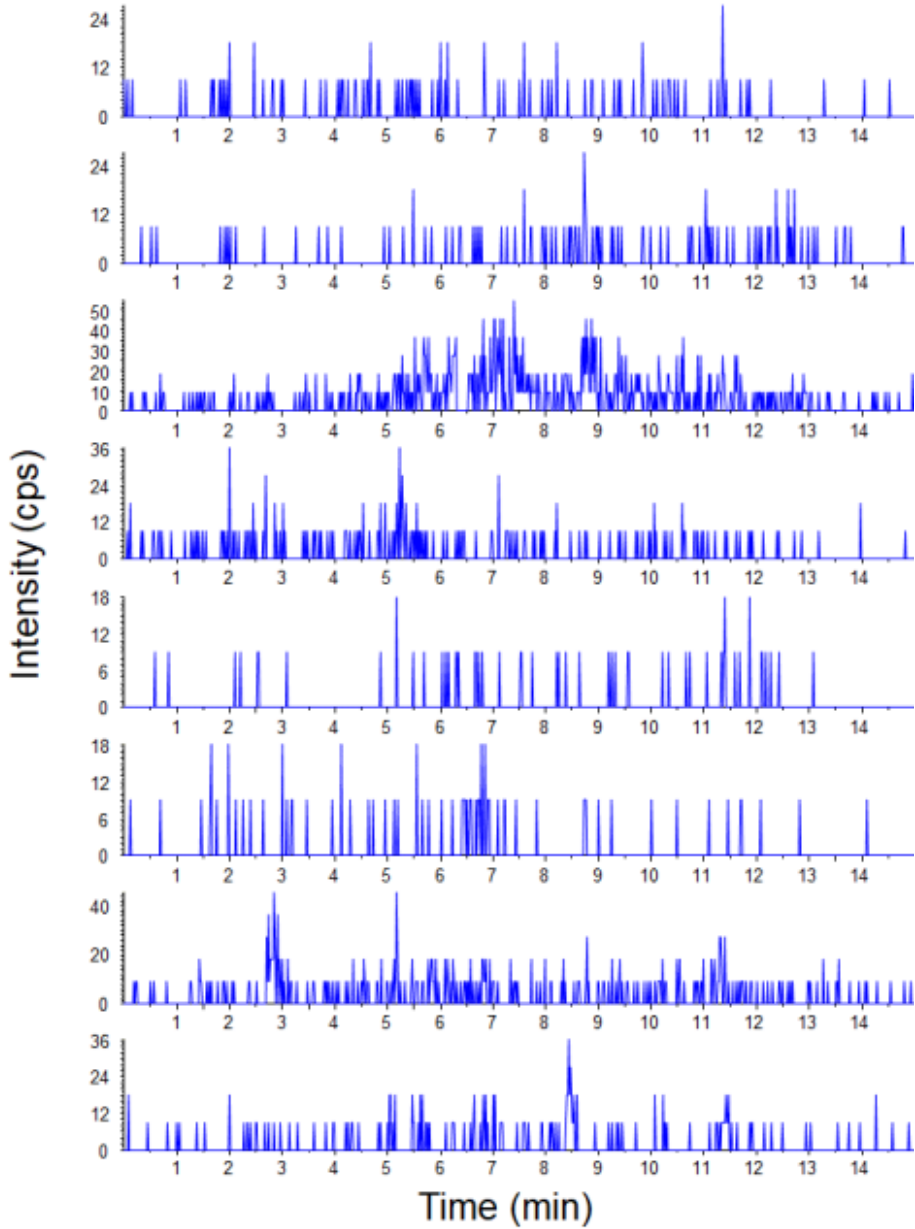
The analytes in the plasma at a concentration of 250 ng/mL were diluted 10-fold with blank plasma. Six replicates were analyzed.

Table 9. Alprazolam concentrations in breastmilk and plasma obtained from a lactating patient, and parameters to assess drug transfer into breastmilk

Time after delivery	Maternal intake dose (mg/day)	Timing of sampling	Total concentration (ng/mL)		M/P ratio	RID (%)	Free drug concentration (ng/mL)		Protein binding (%)
3 days	0.8	Trough	Plasma	5.36	0.52	3.11	Plasma	0.797	85.1
			Milk	2.78			Milk	1.51	45.7
		Peak (2 h)	Plasma	6.95	0.49	3.81	Plasma	1.04	85.0
			Milk	3.4			Milk	1.74	48.8
1 month	1.0	Peak (2 h)	Plasma	13.3	0.41	4.61	Plasma	2.15	83.8
			Milk	5.42			Milk	3.28	39.5

Fig. 1

(A)



(B)

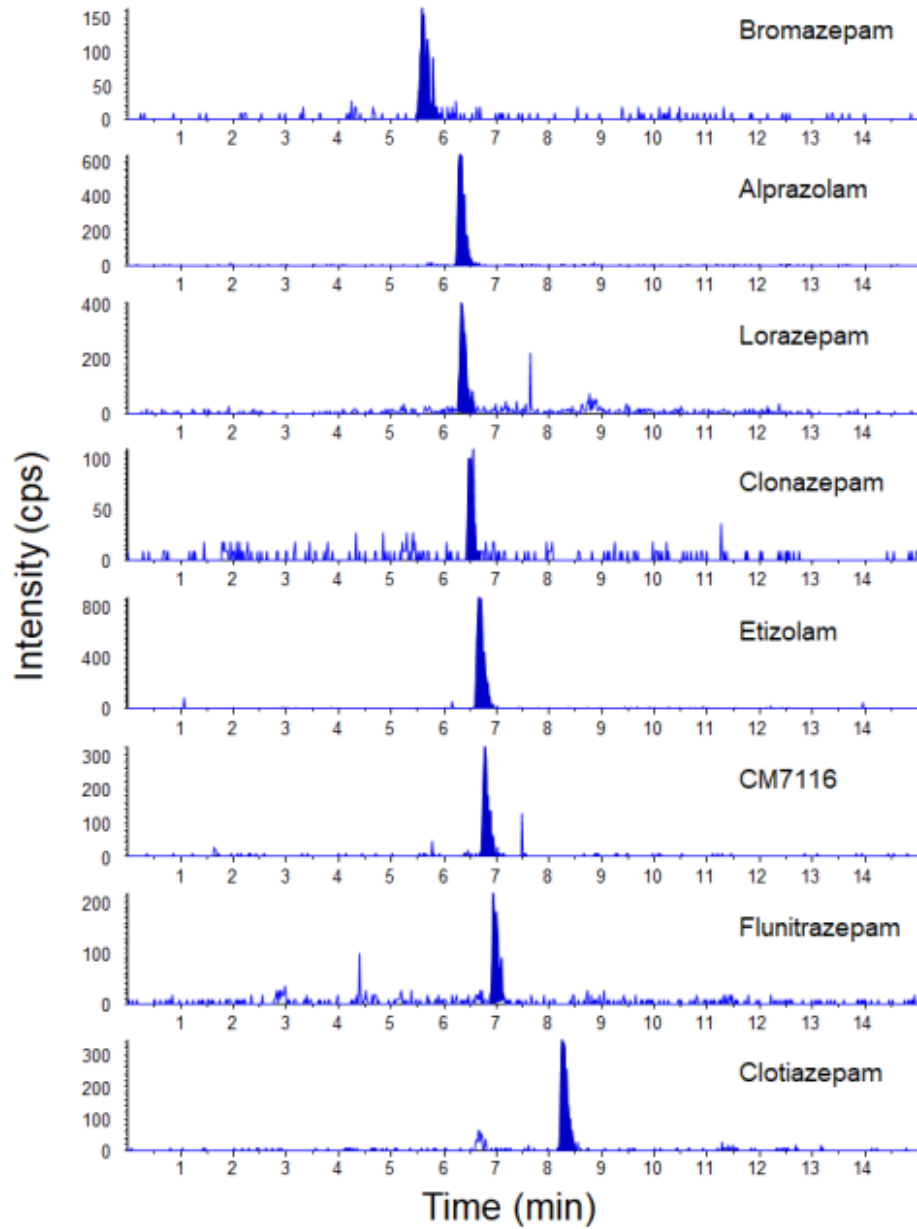
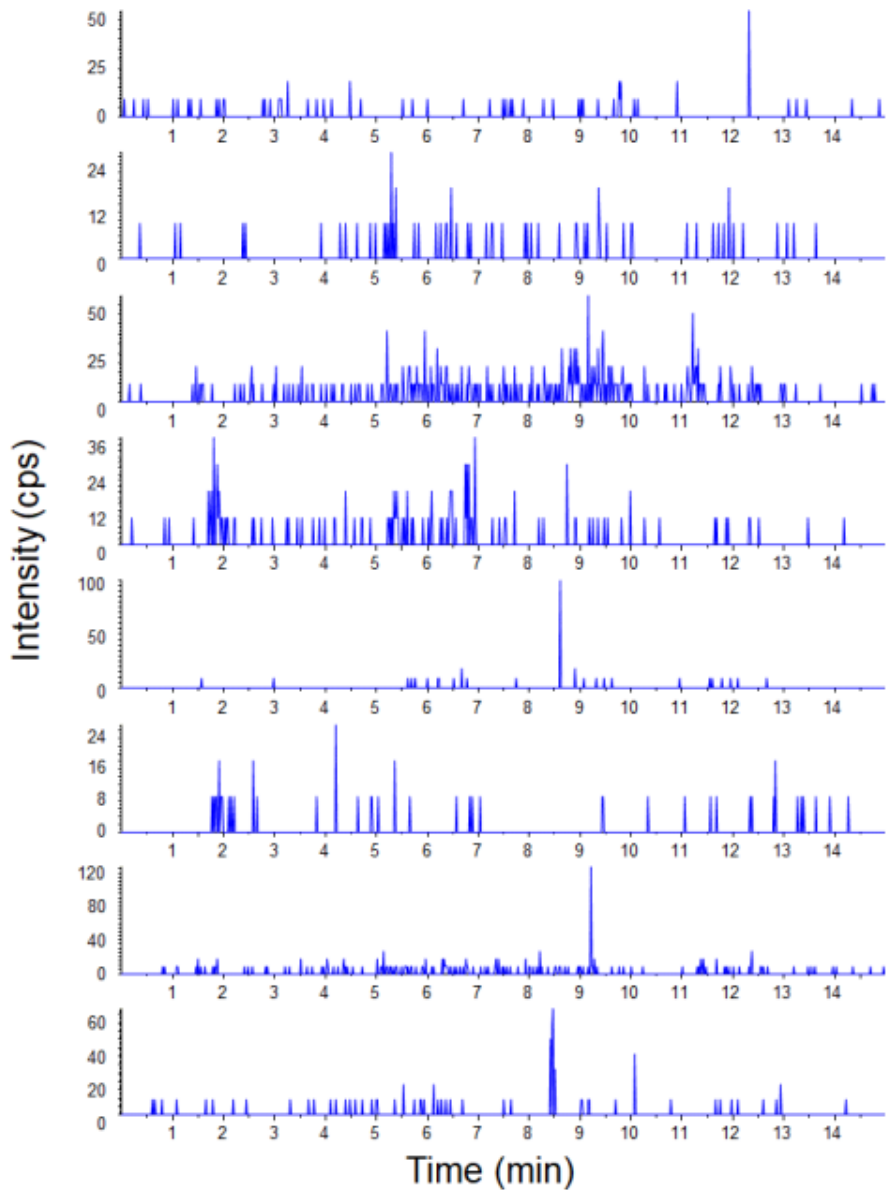


Fig. 1

(C)



(D)

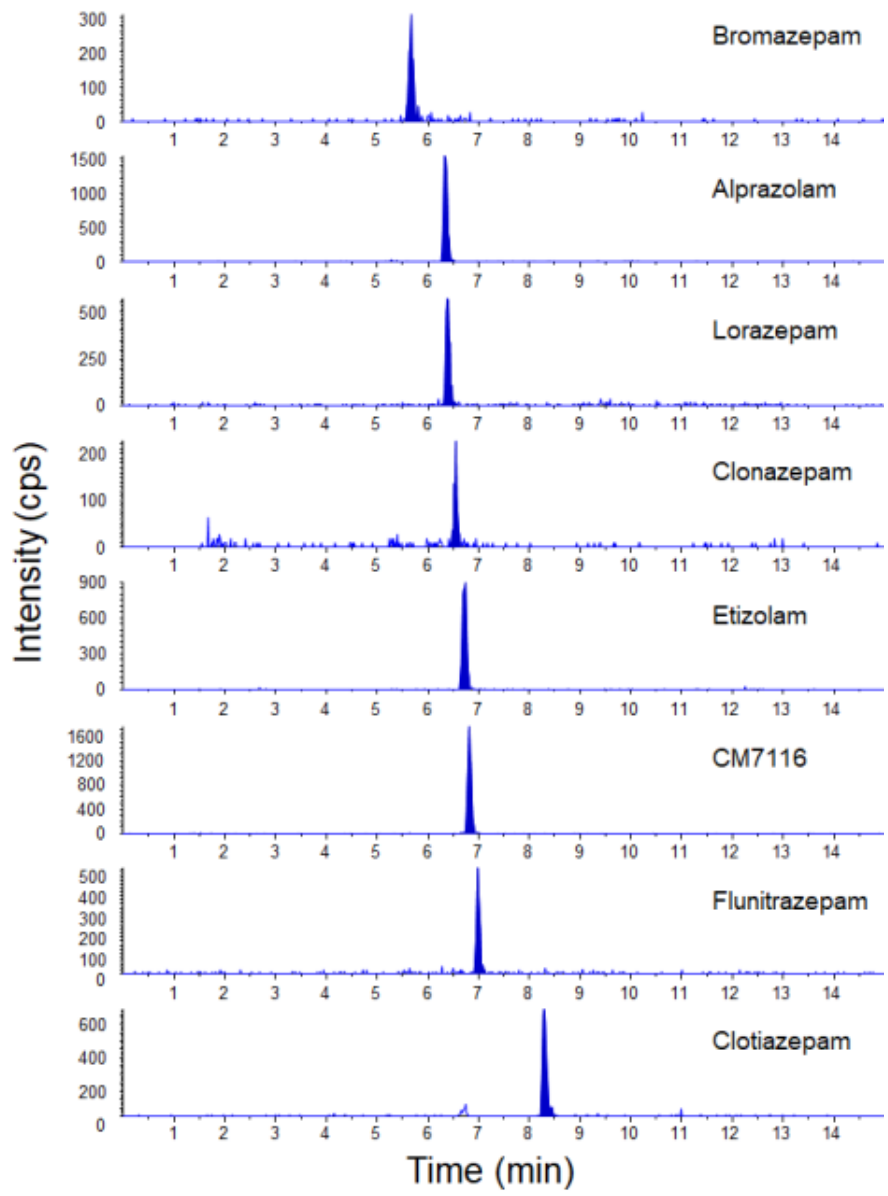
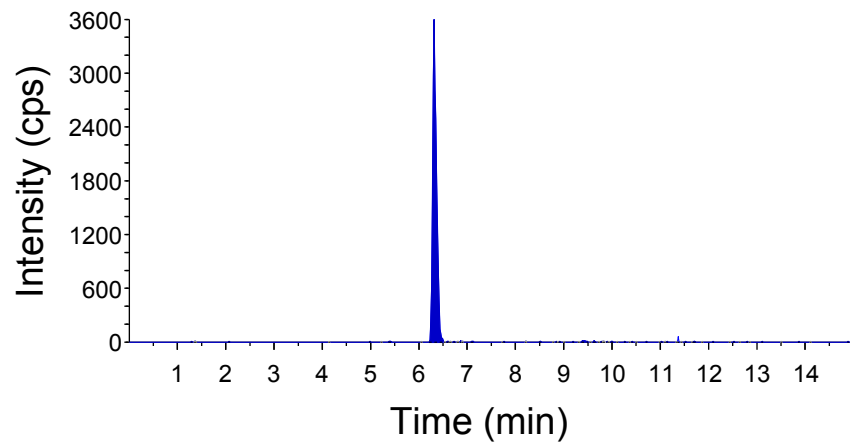
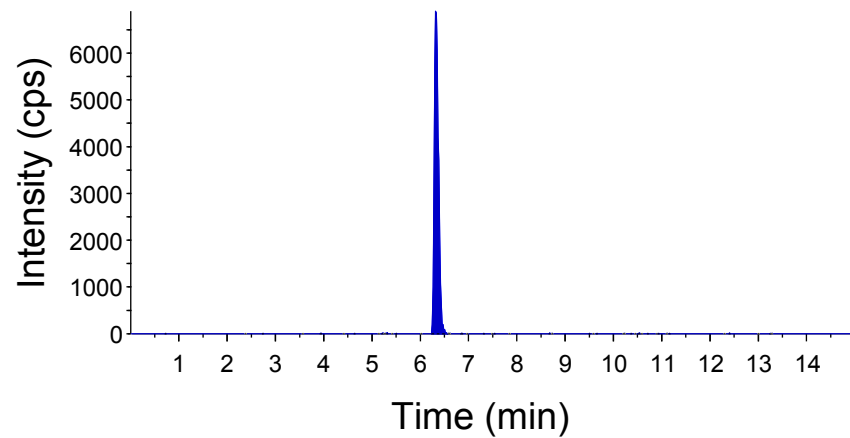


Fig. 2

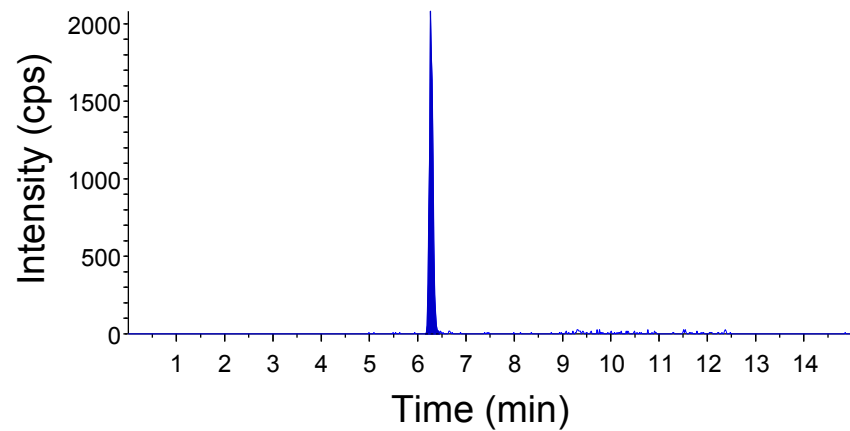
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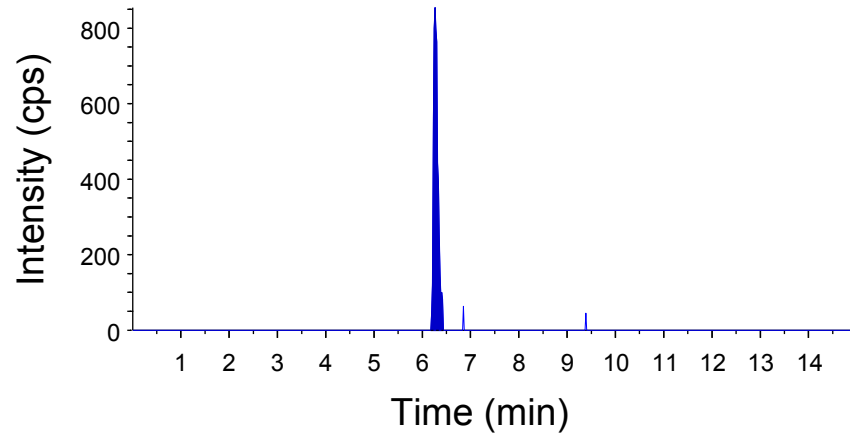
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(C)



(D)



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Quantification of eight benzodiazepines in human breastmilk and plasma by liquid-liquid extraction and liquid-chromatography tandem mass spectrometry: Application to evaluation of alprazolam transfer into breastmilk

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