



Title	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.
Author(s)	Furugen, Ayako; Nishimura, Ayako; Kobayashi, Masaki; Umazume, Takeshi; Narumi, Katsuya; Iseki, Ken
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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.

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

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15 Ayako Furugen^a, Ayako Nishimura^b, Masaki Kobayashi^{b*}, Takeshi Umazume^c, Katsuya Narumi^a,
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17 Ken Iseki^{ab*}
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23
24 ^aLaboratory of Clinical Pharmaceutics & Therapeutics, Division of Pharmasciences, Faculty of
25
26 Pharmaceutical Sciences, Hokkaido University, Kita-12-jo, Nishi-6-chome, Kita-ku, Sapporo 060-
27
28 0812, Japan
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31
32 ^bDepartment of Pharmacy, Hokkaido University Hospital, Sapporo, Japan
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35 ^cDepartment of Obstetrics, Hokkaido University Hospital, Sapporo, Japan
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38 *Corresponding authors
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40 Masaki Kobayashi, Ph.D. Phone/Fax: +81-11-706-3772/3235. E-mail: masaki@pharm.hokudai.ac.jp
41

42 Ken Iseki, Ph.D. Phone/Fax: +81-11-706-3770. E-mail: ken-i@pharm.hokudai.ac.jp
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63 **1 Abstract**
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66 **2** Breastfeeding is strongly encouraged for infant and maternal health. Benzodiazepines (BZDs) are
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68 **3** widely prescribed drugs for symptoms, such as anxiety and insomnia, which many women could
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70 **4** experience during the postpartum period. However, limited information is currently available to
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72 **5** evaluate the transfer of different BZDs into breastmilk. In order to assess the proprieties of this
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74 **6** medication during breastfeeding, robust and sensitive analytical methods to quantify BZDs are
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76 **7** required. For this purpose, we developed a method for quantification of BZDs, including alprazolam,
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78 **8** bromazepam, clonazepam, clotiazepam, etizolam, flunitrazepam, lorazepam, and CM7116 (a
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80 **9** metabolite of ethyl loflazepate), in human breastmilk and plasma using liquid
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82 **10** chromatography/tandem mass spectrometry (LC/MS/MS). Sample preparation was performed by a
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84 **11** simple liquid-liquid extraction (LLE) with ethyl acetate. For sample preparation of CM7116, the
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86 **12** pretreatment process to completely obtain the metabolite was added before the LLE step. The BZDs
87
88 **13** were separated by a C₁₈ column using a gradient elution of acetonitrile in aqueous ammonium
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90 **14** acetate solution, and were detected in the positive ion electrospray mode with multiple reaction
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92 **15** monitoring (MRM). Lower limits of quantification (LLOQs) in breastmilk ranged from 0.25 to 0.5
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94 **16** ng/mL, and those in plasma ranged from 0.5 to 1.0 ng/mL. The intra-day and inter-day precision, and
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96 **17** accuracy of data were assessed and found to be acceptable. The developed method was successfully
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98 **18** applied to measure the concentration of alprazolam in breastmilk and plasma, which were donated by
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100 **19** a lactating woman who had been regularly treated with alprazolam. Milk to plasma (M/P) ratios were
101
102 **20** calculated as 0.52 (before oral administration) and 0.49 (2 h after administration) 3 days after
103
104 **21** delivery. The M/P ratio 1 month after delivery was calculated as 0.41 (2 h after administration). We
105
106 **22** estimated that the relative infant dose (RID) values of alprazolam ranged from 3.11 to 4.61%.
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109 **23**
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111 **24 Keywords:** benzodiazepines, breastmilk, plasma, LC/MS/MS, alprazolam.
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- 1 **Abbreviations:** BZD, benzodiazepines; IS, internal standard; LC/MS/MS, liquid
2 chromatography/tandem mass spectrometry; LLE, liquid-liquid extraction; LLOQ, lower limit of
3 quantification; R.E., relative error; RID, relative infant dose; R.S.D., relative standard deviation;
4 MRM, multiple reaction monitoring

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181 **1. Introduction**
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183 2 Breastfeeding is highly recommended because of various benefits for the health of both
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185 3 mother and breastfed infant, as well as good nutrition. For example, reduction of the risk of
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187 4 development of ovarian and breast cancers, and diabetes in lactating mothers have been reported [1].
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189 5 For breastfed infant, positive effects on intelligence quotient and the development of cognitive ability
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191 6 and prevention of various diseases (e.g., infection, diabetes), have been reported [1]. However,
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193 7 breastfeeding in infancy should be tightly controlled if the mother is medicated. Drugs are thought to
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195 8 be transferred into breastmilk to some extent by passive diffusion and carrier-mediated mechanisms
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198 9 [2]. In general, drugs with low ionization, low plasma protein binding, low molecular weight, and
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200 10 high lipophilicity tend to be transferred into breastmilk [2]. Information on the properties of specific
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202 11 drug transfer into breastmilk and safety is important for adequately encouraging women to breastfeed.

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204 12 Anxiety and insomnia are commonly occurring conditions among pregnant and postpartum
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206 13 women [3]. Untreated maternal anxiety-related illness and abrupt discontinuation of psychotropic
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208 14 drugs could lead to negative effects [4]. Therefore, evaluation of the transfer of psychoactive drugs
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210 15 into breastmilk and the effects of these drugs on breastfed infants are important both for maternal and
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212 16 infant health. Benzodiazepines (BZDs) act as positive allosteric modulators of GABA_A receptor, and
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214 17 are widely prescribed hypnotic, anxiolytic, muscle relaxant, and anticonvulsant drugs. Rubin *et al.*
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216 18 estimated that the adverse event rates of breastfed infants were 17% (1 out of 6), 22% (2 out of 9),
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218 19 and 50% (1 out of 2) when exposed to alprazolam, diazepam, and clonazepam, respectively, after an
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220 20 electronic search study [5]. Kelly *et al.* reported that infant sedation was identified in only 1.6% (2
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222 21 out of 124) of the infants exposed to BZDs by a telephonic follow-up study [6]. The lack of
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224 22 information on the concentrations of the drugs in breastmilk was considered as one of the major
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226 23 limitations of these studies. BZDs have low molecular weight, high lipophilicity, and high plasma
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229 24 protein binding ratio as their common characteristics [7-9]. However, these characteristics differ to
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231 25 some extent among the different BZDs and the half-life of each BZD also varies [7-9]. Currently,
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233 26 detailed data of transfer of each BZD into breastmilk are limited. Therefore, investigation of the
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240 1 transfer of BZDs into breastmilk and methods for quantifying these drugs are significant for correct
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242 2 guidance of breastfeeding in woman under medication.
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244 3 Liquid chromatography/tandem mass spectrometry (LC/MS/MS) is widely used in the
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246 4 quantification of various drugs owing to its high sensitivity, specificity, and capability for
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248 5 simultaneous analyses. Several groups have developed LC/MS/MS methods for the analysis of BZDs
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250 6 in various biological matrix [10], including blood samples (plasma, serum, whole blood) [11-18],
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252 7 urine [17-20], hair [21], and oral fluid [22]. Most of these methods were developed for application in
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254 8 forensic and toxicological sciences. However, only a few methods are currently available for
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256 9 quantifying BZDs in breastmilk samples using LC/MS/MS. Recently, López-García *et al.* reported a
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258 10 method to quantify 40 legal and illegal psychoactive drugs, including several BZDs (alprazolam,
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260 11 diazepam, lorazepam, oxazepam, lormetazepam, temazepam) in breast and bovine milk [23].
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262 12 Although the group successfully applied the method to quantify caffeine in human breastmilk, the
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264 13 reported method was not validated and was not applied for the quantification of BZDs.
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267 14 The purpose of this study was to develop a simple and robust analytical method using
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269 15 LC/MS/MS to measure BZDs in human breastmilk and plasma. In the present study, we have chosen
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271 16 the BZDs which are mainly prescribed for the treatment of anxiety. As analytes, we included eight
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273 17 BZDs namely, alprazolam, bromazepam, clonazepam, clotiazepam, etizolam, flunitrazepam,
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275 18 lorazepam, and CM7116 (a metabolite of ethyl loflazepate). The drugs chosen in the present study
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277 19 were prescribed to the breastfeeding women by the Obstetrics Department of Hokkaido University
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279 20 Hospital (unpublished data). Ethyl loflazepate is a drug, which is approved in approximately 10
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281 21 countries including Japan. The drug is widely used in the Japanese population [24]. After intestinal
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283 22 absorption, the drug is immediately and completely transformed to an unstable metabolite (M-1,
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285 23 CM6913), which is then partially decarboxylated to another metabolite (M-2, CM7116) [25, 26]. It
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287 24 has been reported that ethyl loflazepate is not detected in the plasma after oral administration.
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289 25 Therefore, we developed a quantification method for ethyl loflazepate as a metabolite (CM7116).
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291 26 Using this method, we investigated the levels of alprazolam in human breastmilk (colostrum and
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299 1 mature milk) and plasma obtained from a patient, and successfully evaluated the transfer of the drug
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301 2 into breastmilk. Furthermore, free concentrations of alprazolam were estimated using the ultrafiltrate
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303 3 samples. This was because BZDs show high plasma protein binding ratio [8, 9], which is an
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305 4 important factor affecting the transport of these drugs into breastmilk.
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309 6 **2. Materials and methods**

310 7 **2.1. Ethics**

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312 8 The study was reviewed and approved by the ethics committees of Hokkaido University
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314 9 Hospital (017-0131).
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319 10 **2.2. Chemicals and reagents**

320 11 Alprazolam (purity $\geq 98.0\%$), bromazepam (purity $\geq 99.0\%$), clonazepam (purity $\geq 99.0\%$),
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322 12 clotiazepam (purity $\geq 98.0\%$), etizolam (purity $\geq 98.0\%$), flunitrazepam (purity $\geq 99.0\%$), lorazepam
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324 13 (purity $\geq 98.0\%$) and CM7116 (7-Chloro-5-(2-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-
325
326 14 one)) were purchased from Wako (Tokyo, Japan). Etizolam-d₃ was purchased from Sigma-Aldrich
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328 15 (St. Louis, MO, USA). HPLC-grade organic solvents (acetonitrile, ethyl acetate, and methanol) were
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330 16 purchased from Wako. HPLC-grade aqueous ammonium acetate solution (1 M) was obtained from
331
332 17 Nacalai Tesque (Kyoto, Japan).
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338 18 **2.3. Preparation for calibration curve and quality control (QC)**

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340 19 Standard stock solutions containing mixtures of alprazolam, bromazepam, clonazepam,
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342 20 clotiazepam, etizolam, flunitrazepam, and lorazepam were prepared in methanol (10, 20, 40, 100,
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344 21 200, 400, 1000, 2000, 4000, and 10000 ng/mL). CM7116 standard stock solutions were prepared in
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346 22 methanol (10, 20, 40, 100, 200, 400, 1000, 2000, and 4000 ng/mL). An internal standard (IS) stock
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348 23 solution containing etizolam-d₃ (1 $\mu\text{g/mL}$) was also prepared in methanol. All stock solutions were
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350 24 stored at -80°C . The calibration standards and quality control (QC) samples were prepared at the
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358 1 time of assay by appropriate dilution of the stock solutions in 100 μ L of blank plasma or breastmilk.
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360 2 The concentrations of the calibration standards in breastmilk were 0.25, 0.5, 1, 2.5, 5, 10, and 25
361
362 3 ng/mL for alprazolam; 0.5, 1, 2.5, 5, 10, 25, 50, and 100 ng/mL for bromazepam, clonazepam,
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364 4 etizolam, and lorazepam; and 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, and 100 ng/mL for clotiazepam,
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366 5 flunitrazepam, and CM7116. The concentrations of the calibration standards in plasma were 0.5, 1,
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368 6 2.5, 5, 10, and 25 ng/mL for alprazolam and lorazepam; 0.5, 1, 2.5, 5, 10, 25, 50, 100, and 250
369
370 7 ng/mL for bromazepam, clonazepam, and flunitrazepam; 0.5, 1, 2.5, 5, 10, 25, 50, and 100 ng/mL for
371
372 8 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
373
374 9 ng/mL for CM7116. The concentrations of the QC samples in breastmilk were 0.25, 0.5, 1, and 10
375
376 10 ng/mL for alprazolam; 0.5, 1, 10, and 100 ng/L for bromazepam, clonazepam, etizolam, and
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378 11 lorazepam; and 0.25, 0.5, 10, and 100 ng/mL for clotiazepam, flunitrazepam, and CM7116. The
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380 12 concentrations of the QC samples in plasma were 0.5, 1, 5, and 25 for alprazolam, etizolam, and
381
382 13 lorazepam; 0.5, 1, 25, and 250 for bromazepam, clonazepam, and flunitrazepam; 0.5, 1, 10, and 100
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384 14 clotiazepam; and 1, 2.5, 50, and 100 ng/mL for CM7116.
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388 15 **2.4. Sample pretreatment**

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391 16 Sample was prepared by liquid–liquid extraction (LLE) method. For quantification of
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393 17 CM7116 (a metabolite of ethyl loflazepate), 50 μ L of 0.5 N HCl was added to 100 μ L of each
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395 18 sample to convert an unstable metabolite CM6913 (M-1) to CM7116 (M-2). The samples for
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397 19 quantification of CM7116 were kept for 30 min at room temperature. Then, the sample was
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399 20 neutralized by adding 25 μ L of 1 N NaOH, and 10 μ L of IS solution was added. After treatment, 10
400
401 21 μ L of IS solution was added to the samples and LLE was performed as described below. For
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403 22 quantification of BZDs expect for CM7116, 10 μ L of IS solution was added to 100 μ L of plasma or
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405 23 breastmilk sample. Subsequently, to each sample, 100 μ L of borate buffer (pH 9, 0.1 M) was added
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407 24 and mixed. For extraction, 1500 μ L of ethyl acetate was added to the sample and vortexed for 10 min.
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409 25 The sample was centrifuged at $3,900 \times g$ for 15 min at 4 $^{\circ}$ C. The upper organic layer was carefully
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417 1 collected and dried under a nitrogen gas stream at 40 °C. The sample was reconstituted in 100 µL of
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419 2 mobile phase (acetonitrile:10 mM ammonium acetate solution, 30:70, v/v) and filtrated with a
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421 3 DISMIC-13HP filter (0.2 mm, ADVANTEC, Tokyo, Japan). Ten micro liters of sample was injected
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423 4 to LC/MS/MS.
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426 5 **2.5. LC/MS/MS analysis**

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429 6 Chromatographic separation was carried out using a Shimadzu Prominace 20A System
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431 7 (Shimadzu, Kyoto, Japan) and an Inertsustain C18 column (2.0 × 150 mm, 3 µm GL Science Inc.,
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433 8 Tokyo, Japan). A binary mobile phase consisted of acetonitrile and 10 mM ammonium acetate (pH
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435 9 6.8) solution was flown through the apparatus at a rate of 0.2 mL/min. The acetonitrile composition
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437 10 of the mobile phase was increased from 30% to 90% in a linear gradient over 6 min and maintained
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439 11 at 90% for the first 9.0 min. Acetonitrile composition was then decreased to 30% from 9.0 min to 9.5
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441 12 min and maintained at 30% until 15 min. The column temperature was maintained at 40 °C. The total
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443 13 run time was 15 min. Positive ion electrospray (ESI)-MS/MS analysis was performed using an API
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445 14 3200™ LC/MS/MS System with multiple reaction monitoring (MRM) (Applied Biosystems, Foster
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447 15 City, CA). MRM was performed by monitoring the transitions summarized in Table 1. Parameter
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449 16 settings were as follows: source temperature of 600°C, spray voltage of 5500 V, curtain gas of 30 psi,
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451 17 ion source gas1 of 40 psi, ion source gas2 of 50 psi, and collision gas of 6 arbitrary units. Data were
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453 18 acquired and analyzed using Analyst software (Applied Biosystems).
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457 19 **2.6. Method validation**

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459 20 The present method was validated in accordance with the guidelines (FDA, Guidance for
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461 21 Industry: Bioanalytical Method Validation (2013) and EMEA, Committee for Medicinal Products for
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463 22 Human Use, Guideline on Bioanalytical Method Validation (2011)). For method validation,
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465 23 individual breastmilk from four normal female donors was purchased from Lee BioSolutions
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467 24 (Maryland Heights, MO). A lot (No. 1) was used for all validation assays, calibration curve,
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476 1 precision and accuracy, recovery, matrix effect, stability, and carry-over. Three other lots (No. 2 - 4)
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478 2 were used for matrix effect assessment. In addition, blank breastmilk was provided by two healthy
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480 3 volunteers. Pooled plasma from normal human donors was obtained from Cosmo Bio (Tokyo, Japan)
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482 4 and used for method validation including calibration curve, precision and accuracy, recovery,
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484 5 stability, carry-over, and dilution integrity. For the assessment of matrix effect, individual plasma
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486 6 from six normal female donors was purchased from Cosmo Bio (three lots) and Lee BioSolutions
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488 7 (three lots).
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492 8 ***2.6.1. Linearity of calibration curves***

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494 9 Calibration curves were constructed using stock solutions in 100 μ L of blank breastmilk or
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496 10 plasma in the ranges listed in Table 3 and Table 4. Calibration curves consisted of at least six
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498 11 concentrations. The samples were pretreated as described in section 2.4 and analyzed. Calibration
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500 12 curves were constructed by plotting the peak area ratio (standard to internal standard) versus the
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502 13 nominal concentration and were fitted using least-squares regression with 1/x weighting.
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506 14 ***2.6.2. Precision, accuracy, lower limit of quantification (LLOQ), and recovery***

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508 15 Intra-day precision and accuracy were assessed by analyzing six replicates at four different
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510 16 concentrations on the same day. Inter-day precision and accuracy were assessed by analyzing the
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512 17 replicates at four different concentrations on nine different days. The replicates were prepared and
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514 18 analyzed. The replicates were prepared and analyzed. The R.E. (%) was calculated as [(found
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516 19 concentration – theoretical concentration)/theoretical concentration] \times 100 (%). The precision was
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518 20 obtained as the relative standard deviation (R.S.D.). The acceptable limit for accuracy and precision
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520 21 was $\leq \pm 15\%$ except for LLOQ, for which the acceptable limit was $\leq \pm 20\%$. LLOQ was defined as
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523 22 the concentration with a signal-to-noise (S/N) ratio of at least 10 and precision and accuracy data.
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525 23 Recovery was assessed by spiking known amounts of BZDs into blank plasma or blank breastmilk
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535 1 and comparing the peak areas of analytes spiked before sample preparation with those of analytes
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537 2 spiked after sample preparation that represent 100% recovery.
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540 3 **2.6.3. Matrix effects**

543 4 As described above, six plasma samples from healthy female donors were purchased. With
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545 5 regard to breastmilk, two samples from healthy female volunteers and four sample purchased from
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547 6 the seller (Lee BioSolutions) were used for the assay. For each BZD, the matrix effect was assessed
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549 7 by measuring the peak area in the presence of matrix (peak area of the analyte spiked after sample
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551 8 preparation), and the peak area in the absence of matrix (peak area of an equivalent amount of
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553 9 analyte prepared in the mobile phase). The matrix effect was calculated using the following equation;

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556 10
$$\text{Matrix effect (\%)} = \left(\frac{\text{Peak area in the presence of matrix}}{\text{Peak area in the absence of matrix}} \times 100 \right) - 100$$

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558 11 Furthermore, the accuracy and precision in multiple lots were assessed. For assessment in
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560 12 breastmilk, known amounts of BZDs were spiked in four lots of breastmilk purchased from Lee
561
562 13 BioSolutions (Lot.1 - 4). The samples were prepared as described in Section 2.4, then analyzed. The
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564 14 concentration of the analytes in each lot was quantified using a calibration curve prepared for Lot.2.
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566 15 For assessment of plasma, three lots of plasma obtained from Cosmo Bio (Lot.1 - 3) were assessed.
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568 16 The calibration curves were prepared in pooled plasma
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571 572 17 **2.6.4. Stability**

574 18 The stability of BZDs in breastmilk and plasma was investigated. The short-term stability
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576 19 was assessed after storing BZDs for 24 h in breastmilk or plasma at 4 °C. The long-term stability was
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578 20 assessed after storing BZDs for 8 weeks in breastmilk or plasma at – 30 °C. Freeze-thaw stability
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580 21 was assessed after three freeze-thaw cycles (–30°C to room temperature).
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594 1 **2.6.5. Carry-over**
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597 2 The carry-over was assessed by injection of a blank sample after injection of the highest
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599 3 calibration sample. The area responses of the blank samples were compared to the mean area
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601 4 response of the LLOQ. The peak area of the blank samples following the highest calibration should
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603 5 not exceed 20% of the peak area of the LLOQ and 5% of the peak area of the IS.
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605

606 6 **2.6.7. Dilution integrity**
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608
609 7 The dilution integrity was assessed with the plasma samples. The analytes in the plasma at a
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611 8 concentration of 250 ng/mL were diluted 10-fold with blank plasma. Six replicates were analyzed,
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613 9 and the accuracy and precision were calculated. According to the EMA guideline, the precision and
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615 10 accuracy should not exceed $\pm 15\%$.
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617

618 11 **2.7. Application to clinical samples**
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620
621 12 Samples for quantification of BZD in breastmilk and plasma were obtained from a lactating
622
623 13 woman who was regularly administrated with alprazolam at Hokkaido University Hospital. The
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625 14 volunteer gave written, informed consent, which was approved by the institutional review board. The
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627 15 volunteer participated in the study three days after the delivery and one month after postnatal
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629 16 checkup. Samples were collected before and 2 h after oral administration of alprazolam. A dose of
630
631 17 0.8 mg/day was administrated for 3 days after delivery, and 1.0 mg/day for 1 month after the delivery.
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633 18 The breastmilk and plasma samples were stored at $-30\text{ }^{\circ}\text{C}$ until analysis. For assessment of protein
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635
636 19 binding of alprazolam, we obtained ultrafiltrates of plasma and breastmilk using Centrifree®
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638 20 Ultrafiltration Devices (Merck Millipore, Tulagreen, Ireland) in accordance with the manufacturer's
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640 21 instruction. For quantification of alprazolam in ultrafiltrate samples, calibration solutions were
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642 22 prepared in 100 μL of ultrafiltrate blank plasma or ultrafiltrate blank breastmilk.
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653 1 M/P ratios were calculated as the total concentration in breastmilk/total concentration in
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655 2 plasma. RID was estimated as:

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657 3
$$\text{RID (\%)} = \frac{\text{Total concentration in breastmilk (mg/mL)} \times \text{Infant intake of breastmilk (mL/kg/day)}}{\text{Maternal intake dose (mg/kg/day)}} \times 100$$

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659
660 4 where “Infant intake of breastmilk (mL/kg/day)” was used the average value “150 (mL/kg/day)”.

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662 5 Protein binding ratio was calculated as [(total drug concentration – free drug concentration)/total
663
664 6 drug concentration] × 100 (%).
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712 1 **3. Results and discussion**

713
714 2 **3.1. LC/MS/MS**

715
716 3 In the present study, ESI-MS/MS (positive mode) was used for detecting the BZDs:
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718 4 alprazolam, bromazepam, clonazepam, clotiazepam, etizolam, flunitrazepam, lorazepam, and
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720 5 CM7116 (a metabolite of ethyl loflazepate). Positive ion mass spectra indicated the presence of the
721
722 6 protonated molecules for each compound (data not shown). Product ions with high intensity were
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724 7 selected for analysis. Since bromazepam has a bromine in the structure, two intense ions (m/z 316
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726 8 and m/z 318) were observed. Although the intensity of m/z 316 > 182 was higher than that of m/z
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728 9 318 > 182, the interfering peak was observed in blank breastmilk when the transition m/z 316 > 182
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731 10 was monitored. Therefore, m/z 318 was selected as a precursor ion and the transition m/z 318 > 182
732
733 11 was monitored for the detection of bromazepam. Table 1 shows the ion pairs selected for MRM and
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735 12 parameter settings. Many studies have monitored multiple transitions with each analyte, whereas
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737 13 several studies have also used a single transition for quantification of BZDs [11, 16]. Monitoring
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739 14 multiple transitions for each analyte is advisable to increase the specificity and reduce the risk of
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741 15 false positives. Although we selected a single transition for quantification of each analyte, no
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743 16 interfering peak was observed in any of the tested lots of blank plasma and blank breastmilk samples.
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745 17 The transitions, as shown in Table 1 were monitored.

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747 18 Various analytical columns, including C18 [11, 13-18, 20-22] and phenyl types [12, 19],
748
749 19 have been applied for the separation of BZDs. In our preliminary study, two types of columns (C18
750
751 20 column or phenyl-hexyl column) were tested. Separation pattern of the analytes between these
752
753 21 columns was not largely different. In the present study, a C18 column (Inertsustain C18, 2.0 × 150
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755 22 mm, 3 μm) was used for simultaneous analysis. Furthermore, several mobile phases, different
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758 23 combinations of organic solvents (methanol and acetonitrile) and aqueous solutions (10 mM
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760 24 ammonium formate plus 0.1% formic acid, 10 mM ammonium acetate, 10 mM ammonium
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762 25 bicarbonate), were tested. When the solution having a lower pH (10 mM ammonium formate plus
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764 26 0.1% formic acid) was tested, the sensitivity of the analytes (bromazepam and lorazepam) was lower

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771 1 than in solutions having a higher pH [10 mM ammonium acetate (pH 6.8) or 10 mM ammonium
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773 2 bicarbonate (pH 8)]. Since sharp peaks were obtained with ammonium acetate rather than with
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775 3 ammonium bicarbonate, ammonium acetate solution was selected for this study. Although both
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777 4 methanol and acetonitrile produced sharp peaks with optimal separation and sensitivity, acetonitrile
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779 5 was selected as an organic solvent because it created lower pressure on the column. Therefore, a
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781 6 gradient elution with 10 mM ammonium acetate and acetonitrile as described in 2.5, was selected for
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783 7 this study. The total run time was 15 min including column equilibration. Figure 1 shows the
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785 8 representative chromatograms of the blank sample (A and C), the blank sample with LLOQ levels of
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787 9 BZDs (B and D). As shown in the figures, no significant interference was observed in the blank
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789 10 sample at the time of retention of each BZD, showing that the method has optimal specificity.
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791 792 11 793 794 12 **3.2. Sample preparation**

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796 13 Several developed methods quantify BZDs in plasma, including ethyl loflazepate, as a
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799 14 parent drug [11, 17]. As mentioned in the *Introduction* section, ethyl loflazepate is immediately
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801 15 transformed to an unstable carboxylic metabolite (M-1, CM6913), which is then partially
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803 16 decarboxylated to another metabolite (M-2, CM7116) [25, 26]. Sample treatment for completely
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805 17 changing to CM7116 was based on the previous reported method with some modifications [25]. As
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807 18 described in 2.4, plasma and breastmilk sample for quantifying CM7116 was first treated with HCl
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809 19 and then neutralized with NaOH before LLE.

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811 20 Several methods for sample preparation of BZDs, including LLE [12-15, 22], solid-phase
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813 21 extraction [11, 16, 17], protein precipitation [20], have been reported. In the present study, a simple
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815 22 LLE method was applied for the quantification of the eight BZDs. Ethyl acetate was the organic
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817 23 solvent used in the extraction. The recovery from breastmilk ranged from 56.5 to 83.8% and that
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819 24 from plasma ranged from 66.6 to 116.7% (Table 2). Compared to the previously described methods
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821 25 quantifying BZDs such as alprazolam bromazepam, clonazepam, flunitrazepam, and lorazepam in
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830 1 plasma [13-15, 17, 18], a smaller sample volume was needed for the present method (100 μ L) to
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832 2 have good sensitivity. In their method, Simonsen *et al.* [15] and Verplaetse *et al.* [17] have reported
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834 3 the LLOQs of alprazolam, bromazepam, clonazepam, flunitrazepam, and lorazepam as 2-5 ng/mL.
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836 4 Marin *et al.* [14] have reported the LLOQs of the same BZDs were 1 ng/mL, whereas Montenarh *et*
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838 5 *al.* [13] have reported that LLOQs of the same BZDs were 5–40 ng/mL. The LLOQs of alprazolam,
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840 6 clonazepam, flunitrazepam, and lorazepam were found to be 1–5 ng/mL in the method by Mata *et al.*
841
842 7 [18]. In the present study, the LLOQ of the same BZDs in plasma was 0.5 ng/mL. The previously
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844 8 reported methods required larger sample volumes (200–500 μ L) than the present method. López-
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846 9 García *et al.* used protein precipitation by methanol for quantification of psychoactive drugs, such as
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848 10 alprazolam and lorazepam, in breastmilk [23]. The LLOQs of alprazolam and lorazepam were 0.5
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850 11 ng/mL and 3.0 ng/mL, respectively. The method used 0.5 mL of breastmilk. A smaller sample
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852 12 volume was needed to carry out the present method compared to the method [23].
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856 857 14 **3.3. Method validation**

858 859 15 **3.3.1. Calibration curve**

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861 16 Calibration standards were constructed by spiking at least six different concentrations of
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863 17 analytes to blank breastmilk or plasma. In the ranges showed in the Table 3 and Table 4, the present
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865 18 method presented good linearity both in breastmilk ($r^2 > 0.997$) and in plasma ($r^2 > 0.993$).
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867 868 19 **3.3.2. Accuracy and precision**

869
870 20 The intra-day precision and inter-day precision as well as accuracy were tested at four
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872 21 different concentrations. The data are summarized in Table 3 (breastmilk) and Table 4 (plasma). In
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874 22 the quantification method with breastmilk, the intra-day precision ranged from 1.3 to 17.7% and the
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876 23 accuracy ranged from – 19.4 to 18.2%. The inter-day precision ranged from 2.5 to 13.2% and the
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878 24 accuracy ranged from – 20.0 to 10.1%. In the quantification method with plasma, the intra-day
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880 25 precision ranged from 2.2 to 12.1% and the accuracy ranged from – 19.2 to 16.6%. The inter-day
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889 1 precision ranged from 1.8 to 14.4% and the accuracy ranged from – 14.3 to 16.2%. The precision and
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891 2 accuracy were within 15%, except for LLOQ (those of LLOQ were within 20%). These results
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893 3 indicate that the present method is highly reliable and has good accuracy and precision.
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895 4 **3.3.3. Matrix effect**

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898 5 The results of the matrix effect assessment are summarized in Table 5. Ion suppression was
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900 6 observed with most of the analytes, especially clonazepam in both breastmilk and plasma. In the
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902 7 present study, we used deuterium-labeled etizolam as the internal standard for all analytes. Therefore,
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904 8 we investigated the accuracy and precision in multiple lots of breastmilk and plasma to assess
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906 9 whether the IS can compensate for variations in matrix effect and recovery. As shown in Table 6, no
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908 10 significant variability among the lots was observed. During quantification of breastmilk, the
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910 11 precision among the lots ranged from 2.2 to 19.6% and the accuracy ranged from – 7.4 to 20.5%.
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912 12 With plasma, the precision among the lots ranged from 0.4 to 19.7% and the accuracy ranged from –
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914 13 11.8 to 17.9%. A single IS for multiple analytes was used in some studies for quantifying BZDs in
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916 14 plasma and urine [11, 12, 16, 17, 21, 22]. In the present method, a single IS was used as a surrogate
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918 15 IS because of the chemical similarity with BZDs. Considering the validation data (Table 3, 4, and 6)
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920 16 and clinical concentration of BZDs [27], we concluded that the use of a single IS was adequate to
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922 17 quantify BZDs in the present study. The use of an isotopically stable IS for each analyte is, however,
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924 18 generally recommended for LC/MS/MS analysis. In some conditions, for e.g. for detection of a
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926 19 concentration of 1 ng/mL of alprazolam in breast milk, the accuracy and precision were large to
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928 20 some extent. Future studies are needed to assess whether the use of multiple ISs can lead to
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930 21 improvement of accuracy and precision among the lots of matrices.
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933 22 **3.3.4. Stability**

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936 23 We examined the short-term stability of BZDs (for 24 h at 4 °C), long-term stability (for 8
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938 24 weeks at -30 °C), and freeze-thaw stability both in breastmilk and plasma. The data are summarized
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940 25 in Table 7. After storing BZDs in these conditions, the remaining amounts of the compounds were
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948 1 quantified. For short-term stability, the remaining amounts of BZDs in the samples ranged from
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950 2 94.7% to 111.3% in breastmilk, and from 86.0% to 104.0% in plasma. For long-term stability, the
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952 3 remaining amounts of BZDs ranged from 91.2% to 112.5% in breastmilk, and from 92.9% to 118.7%
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954 4 in plasma. After three freeze-thaw cycles, the remaining amounts of BZDs ranged from 82.5% to
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956 5 105.6% in breastmilk, and from 92.7% to 104.4% in plasma. These results indicated that no
957
958 6 significant degradation was observed at least in these conditions.

960 7 **3.3.5. Carry-over**

963 8 The carry-over with all the analytes were within 20% of the LLOQ (range: 0 to 9.7% for
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965 9 breastmilk and 0.4 to 9.7% for plasma). The carry-over with IS was < 0.1%.

968 10 **3.3.6. Dilution integrity**

970 11 The plasma concentration of different analytes (alprazolam, clonazepam, lorazepam, and
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972 12 CM7116) in an authentic sample may exceed the calibration range based on the reported clinical
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974 13 range [27], whereas the breastmilk concentration of the samples is expected to be within the
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976 14 calibration range. Therefore, we assessed the dilution integrity in the plasma sample. As shown in
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978 15 Table 8, accuracy ranged from – 1.9 to 6.0% and the precision ranged from 2.1 to 6.3%. The results
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980 16 indicated that the plasma concentration of all the analytes up to a concentration of 250 ng/mL could
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982 17 be accurately quantified with 10-fold dilution.

986 19 **3.4. Application of the method for the assessment of alprazolam transfer into breastmilk**

989 20 To investigate the suitability of the developed method, the method was applied to quantify
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991 21 alprazolam in breastmilk and plasma samples, which were donated by a lactating woman who had
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993 22 been regularly treated with alprazolam before pregnancy. Samples were collected before (trough)
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995 23 and 2 h after (estimated the time of maximum concentration in plasma) oral administration of
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997 24 alprazolam. Figure 2 shows the chromatograms of the authentic samples. As shown in Figure 2A and
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999 25 2B, alprazolam was detected both in breastmilk and in plasma samples. No peak other than that of
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1007 1 alprazolam (m/z 309 > 281) was observed. The transitions, as shown in Table 1 were monitored (data
1008
1009 2 not shown). The analyzed data are summarized in Table 9. In the present study, the M/P ratio was
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1011 3 calculated to be 0.41 one month after delivery. At 3 days after delivery, M/P ratios were calculated to
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1013 4 be 0.52 (trough) and 0.49 (peak). It has been reported that the time profiles of alprazolam
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1015 5 concentrations in breastmilk and plasma were in a parallel fashion [28]; the study group reported that
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1017 6 the M/P ratio of alprazolam from lactating volunteers 6–28 weeks after single administration of 0.5
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1019 7 mg alprazolam was estimated to be 0.36 ± 0.11 . These results were not significantly different from
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1021 8 the results of previous studies, indicating the applicability of the present method for the evaluation of
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1023 9 transfer of BZDs into breastmilk.

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1025 10 Three days after delivery, M/P ratios tended to be higher than that after one month. It has
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1028 11 been reported that the composition of colostrum varies when it changes to mature milk [29].
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1030 12 Furthermore, it is generally thought that drugs penetrate more in colostrum than in mature milk,
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1032 13 because of the incompleteness of tight junctions of mammary gland cells [2]. The differences
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1034 14 between colostrum and mature milk may affect the M/P ratio. Since there is limited information
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1036 15 about the change over time during lactation, further studies are required to better understand the
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1038 16 properties of BZD transfer into breastmilk.

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1040 17 Furthermore, we estimated the RID values from the concentration of alprazolam in
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1042 18 breastmilk, the results ranged from 3.11 to 4.61%. Generally, if a RID value of a drug is lower than
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1044 19 10%, it is considered to be compatible with breastfeeding [2]; nevertheless, each one of the
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1046 20 individual situations should be taken in account. The present finding may support the propriety of
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1048 21 breastfeeding during alprazolam administration. Several studies have reported adverse effects on
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1050 22 breastfed infants, such as sedation, although the concentration levels have not been investigated [5,
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1052 23 6]. As per Medications and Mothers' Milk 2017 [2], alprazolam is categorized as L3 (Limited Data-
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1054 24 Probably Compatible). In the Drugs in Pregnancy and Lactation (11th edition), the drug is
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1056 25 categorized as "Limited Human Data-Potential Toxicity" [30]. Future studies are urgently needed to
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1058 26 clarify whether the potential adverse effects are related with concentration levels of BZDs.

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1066 1 As described in the *Introduction* part, BZDs showed high plasma protein binding ratio [8, 9],
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1068 2 which is an important factor affecting its transport into breastmilk. Therefore, we quantified
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1070 3 ultrafiltrate samples to investigate the concentration of free alprazolam and to estimate the protein
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1072 4 binding ratio. As shown in Figure 2C and 2D, alprazolam was detected both in ultrafiltrate
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1074 5 breastmilk and ultrafiltrate plasma samples. The percentages of protein binding in plasma ranged
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1076 6 from 83.8 to 85.1% and those in breastmilk from 39.5 to 48.8%. The data of plasma protein binding
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1078 7 was close to the previous reported values [8]. The results indicated that the present method could be
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1080 8 applied for the evaluation of protein binding of BZDs in breastmilk and plasma.
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1085 10 **4. Conclusion**

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1087 11 In the present study, we developed a method for the quantification of eight BZDs, including
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1089 12 alprazolam, bromazepam, clonazepam, clotiazepam, etizolam, flunitrazepam, lorazepam and
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1091 13 CM7116 (a metabolite of ethyl loflazepate), in human breastmilk and human plasma, using
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1093 14 LC/MS/MS. In the present method, only 100 μ L of breastmilk and plasma were used. Sample
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1095 15 preparation was conducted by a simple LLE. For quantification of CM7116, pretreatment process for
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1097 16 completely changing to the metabolite was added. Currently, there are few LC/MS/MS methods for
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1099 17 the quantification of BZDs in breastmilk. To the best our knowledge, there are no reports on the
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1101 18 determination methods for bromazepam, clonazepam, clotiazepam, etizolam, flunitrazepam, and
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1103 19 CM711 in breastmilk sample. Furthermore, the quantification methods for etizolam, clotiazepam,
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1105 20 and CM7116 in plasma are also limited compared to other BZDs. The developed method was
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1107 21 successfully applied to the measurement of alprazolam in authentic samples. We revealed the
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1109 22 concentrations of alprazolam in breastmilk and plasma, which were obtained from a lactating woman
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1111 23 who was regularly administrated alprazolam. Since there is limited information obtained from
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1113 24 patients regularly administrated BZD, the findings of the present study may help to identify a better
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1 therapeutic strategy during breastfeeding. The method described here could be useful for future
2 studies evaluating the properties of BZDs transfer into breastmilk.

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7 **Conflicts of Interest**

8 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.

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Table 2. Recovery of BZDs from breastmilk and plasma

	Concentration (ng/mL)	Recovery (%) (Mean \pm S.D., n=3)					
		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

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	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

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			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
			0.5	0.542	7.7	8.4	0.546	8.0	9.3
Bromazepam	0.5 - 100	0.997	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
			0.5	0.591	17.6	18.2	0.551	12.5	10.1
Clonazepam	0.5 - 100	0.998	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
			0.25	0.260	17.7	3.9	0.243	13.1	-2.6
Clotiazepam	0.25 - 100	0.998	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
			0.5	0.421	5.0	-15.8	0.437	5.0	-12.6
Etizolam	0.5 - 100	0.998	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
			0.25	0.267	7.2	6.6	0.271	12.0	8.2
Flunitrazepam	0.25 - 100	0.998	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
			0.5	0.403	8.2	-19.4	0.400	10.5	-20.0
Lorazepam	0.5 - 100	0.997	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
			0.25	0.246	12.3	-1.5	0.226	12.2	-9.6
CM7116	0.25 - 100	0.998	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 (ng/mL)	R.S.D. (%)	R.E. (%)	Found (ng/mL)	R.S.D. (%)	R.E. (%)
			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

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			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 (ng/mL)	Matrix effect (%)	Concentration (ng/mL)	Matrix effect (%)
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

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	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
CM7116	0.5	-30.5	1	-17.5
	100	-21.5	100	-10.8

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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.
		(ng/mL)	Lot. 1	Lot. 2	Lot. 3	Lot. 4	(%)	(%)	(ng/mL)	Lot. 1	Lot. 2	Lot. 3	(%)	(%)	
	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	24 h	8 weeks	Freeze-thaw	Concentration	24 h	8 weeks	Freeze-thaw
		(ng/mL)	(4 °C)	(-30 °C)	(-30°C and	(ng/mL)	(4 °C)	(-30 °C)	(-30°C and
					room temperature,				room temperature, 3
					3 cycles)				cycles)
2003	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
2004		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
2005	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
2006		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
2007	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
2008		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
2009	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
2010		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
2011	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
2012		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
2013	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
2014		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
2015	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
2016		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
2017	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
2018		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.

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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 (%)
			Plasma	Milk			Plasma	Milk	
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

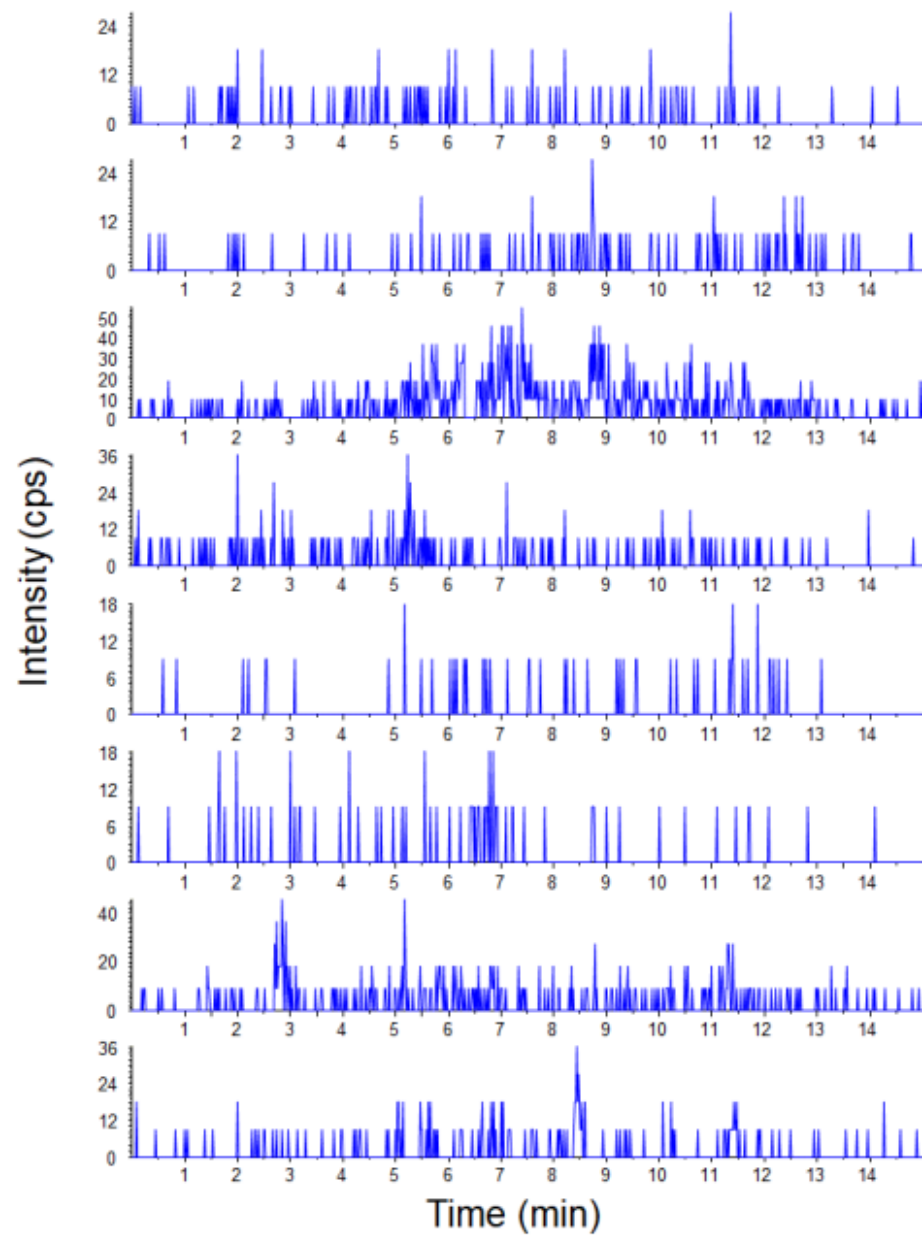
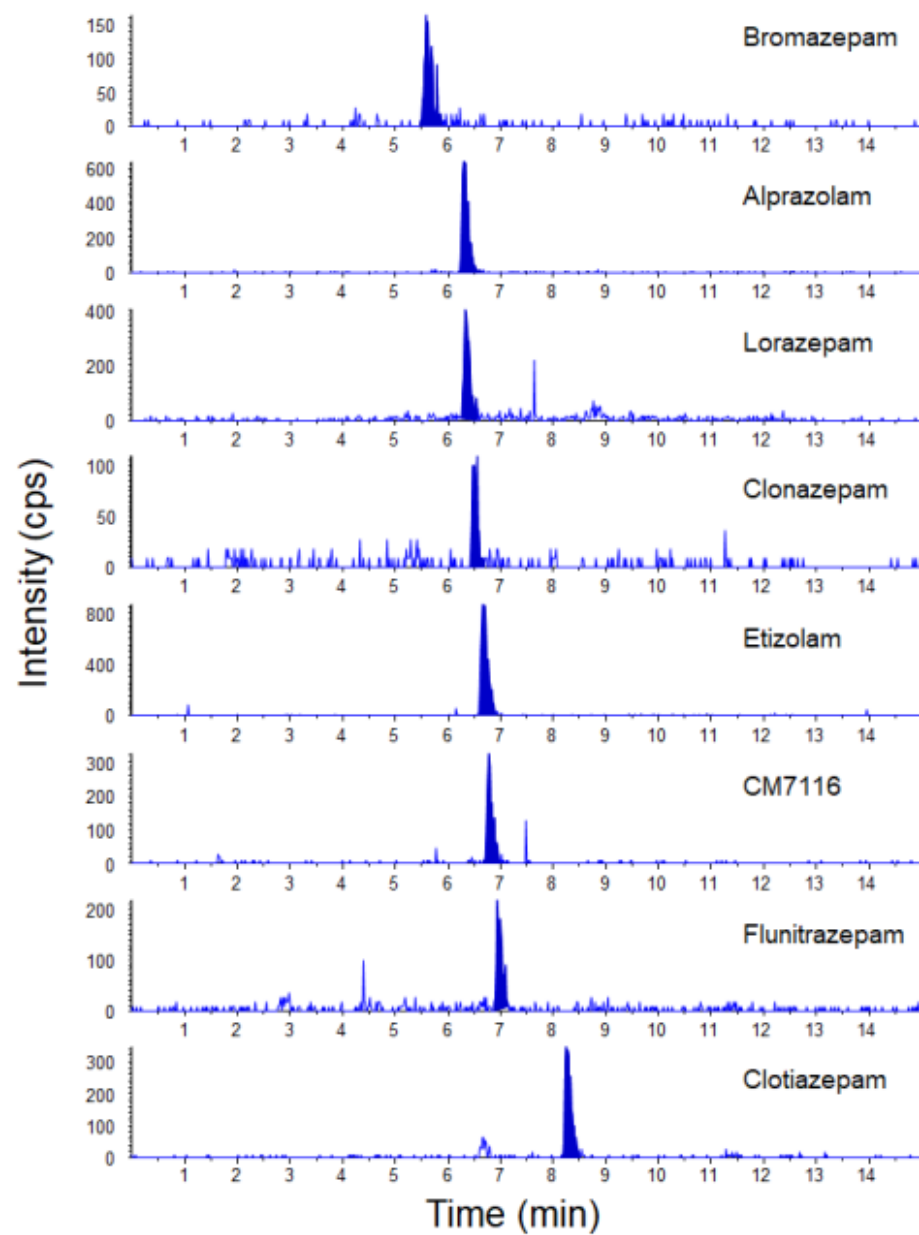
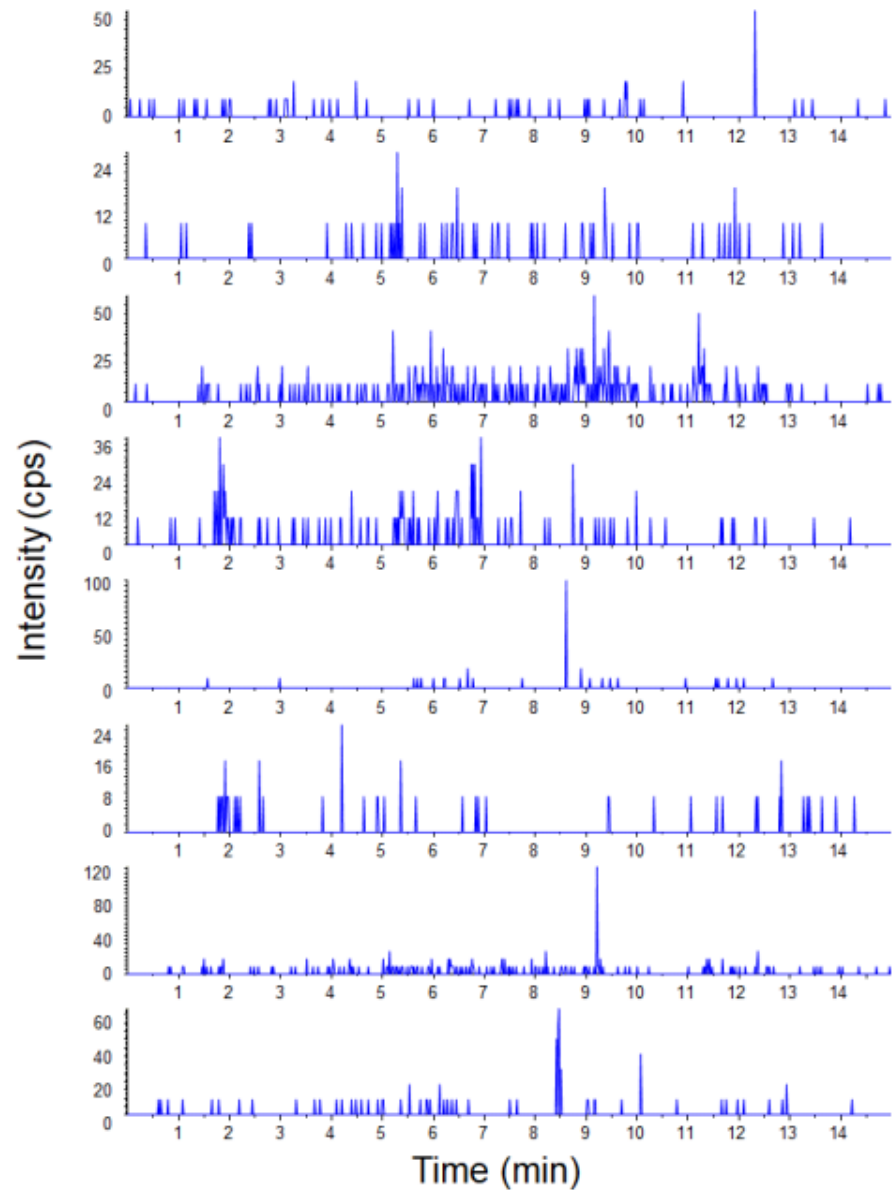
Fig. 1**(A)****(B)**

Fig. 1

(C)



(D)

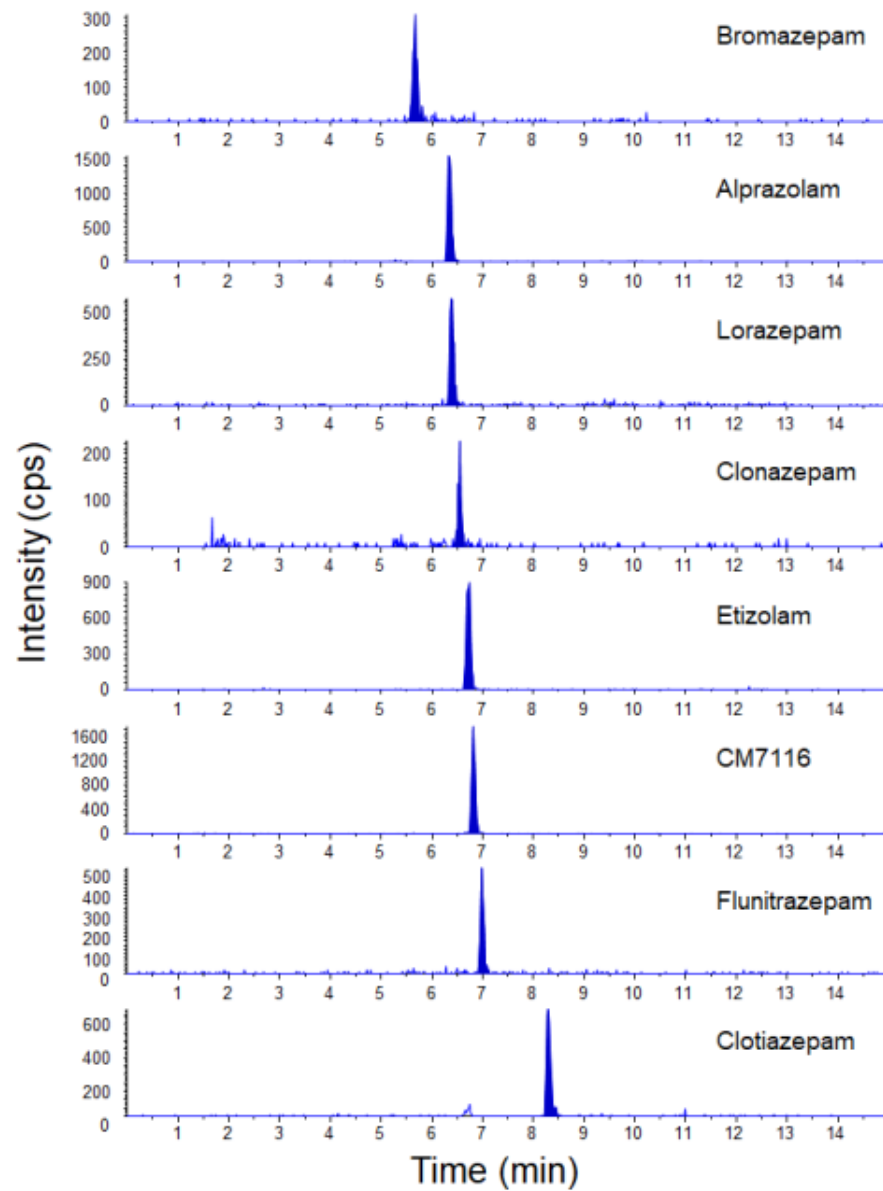
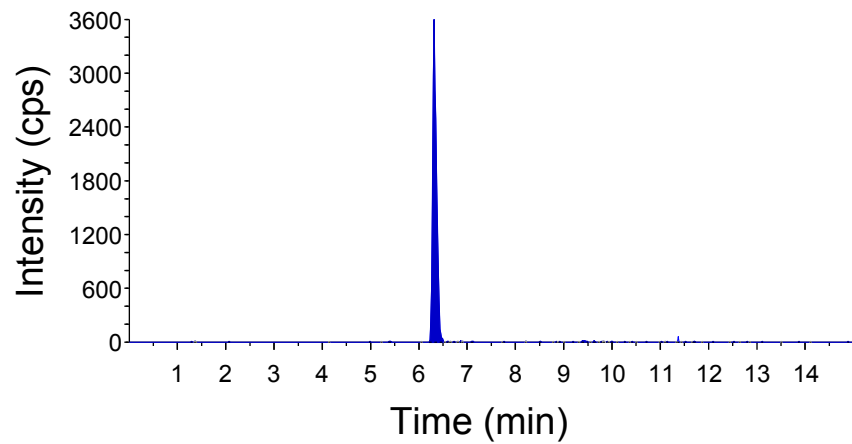
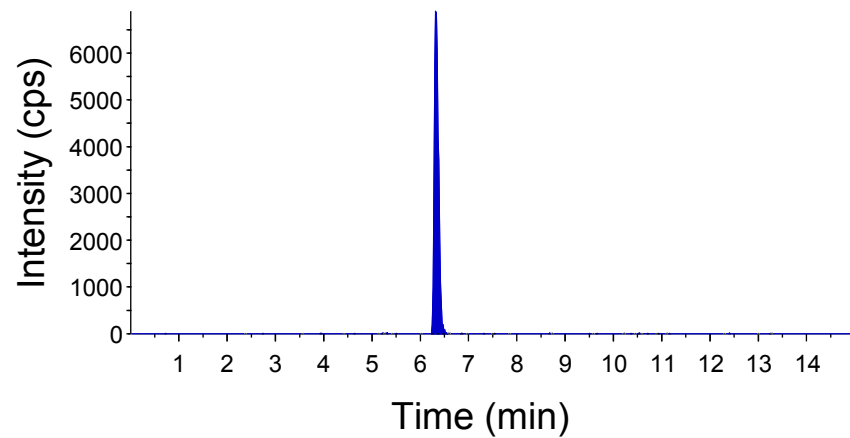


Fig. 2

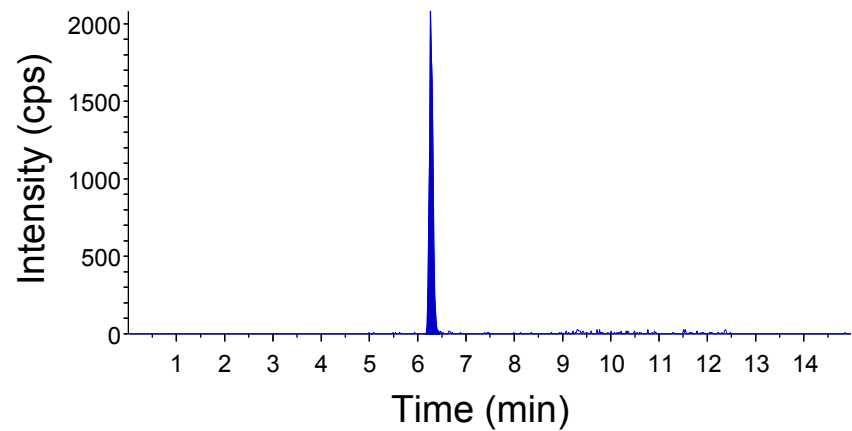
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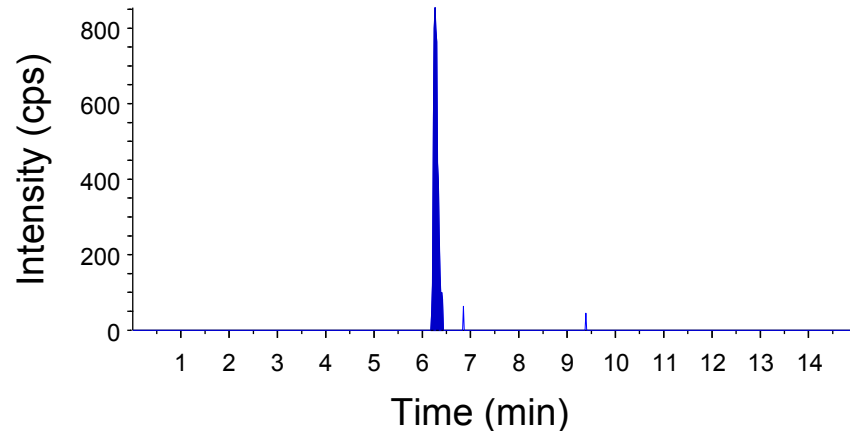
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Quantification of eight benzodiazepines in human breastmilk and plasma by liquid-liquid extraction and liquid-chromatography tandem mass spectrometry: Application to evaluation of alprazolam transfer into breastmilk

AUTHORS

Ayako Furugen, Ayako Nishimura, Masaki Kobayashi, Takeshi Umazume, Katsuya Narumi, Ken Iseki.

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Contact Editage

Worldwide

request@editage.com

+1 877-334-8243

www.editage.com

Japan

submissions@editage.com

+81 03-6868-3348

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Korea

submit-

korea@editage.com

1544-9241

www.editage.co.kr

China

fabiao@editage.cn

400-005-6055

www.editage.cn

Brazil

contato@editage.com

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