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MALDI-TOF mass spectrometric determination of eight benzodiazepines with two of their metabolites in blood

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ABSTRACT

A rapid and sensitive method was developed for the determination of benzodiazepines and benzodiazepine-like substances (BZDs) by matrix-assisted laser desorption ionization (MALDI)-time-of-flight (TOF)-mass spectrometry (MS). In this method, α-cyano-4-hydroxy cinnamic acid was used as the matrix to assist the ionization of BZDs. Determination of 8 BZDs (with two of their metabolites) belonging to top 12 medical drugs detected in poisonous cases in Japan, was performed using diazepam-d_5 as the internal standard. The limit of detection of zolpidem was 0.07 ng/ml with its quantification range of 0.2–20 ng/ml in blood, in the best case, and the limit of detection of flunitrazepam was 2 ng/ml with its quantification range of 6–200 ng/ml in blood, in the worst case. The spectra of zopiclone in MALDI-MS and MS/MS were different from those in electrospray ionization MS and MS/MS. Present method provides a simple and high throughput method for the screening of these BZDs using only 20 μl of blood. The developed method was successfully used for the determination of BZDs in biological fluids obtained from two victims.

1. Introduction

Benzodiazepines and benzodiazepine-like substances (BZDs) interact with γ-aminobutyric acid (GABA) as well as the β-subunit of the GABA receptor in the central nervous system and produce anti-anxiety, hypnotic and sedative effects. They have been commonly prescribed by medical professionals for the treatment of various mental illnesses and are frequently encountered in emergency toxicology screening, drug-abuse testing and forensic medical examination. That is, 8 BZDs such as flunitrazepam, etizolam, triazolam, nitrazepam, brotizolam, zolpidem, bromazepam and zopiclone, in the order of decreasing number of their victims, were found among top 12 medical drugs that were detected in poisonous cases in Japan [1]. For the detection of BZDs, gas chromatography (GC) coupled to mass spectrometry (MS) was used previously [2]. Some of BZDs, however, were labile at high temperature used in GC and some should be derivatized to volatile compounds before the detection by GC–MS (/MS) [2]. Therefore BZDs were detected currently by liquid chromatography (LC)–MS (/MS) [3–9].

Matrix assisted laser desorption ionization (MALDI)-time-of-flight (TOF)-MS is a very popular and powerful tool that is used in the analysis of high-mass biomolecules [10] because the signals of matrices decrease with increasing m/z values, and hence they do not interfere with the signals of high-mass molecules. The MALDI-TOF-MS on low-mass ($m/z < 500$) molecules [11–14] was not performed so often previously. BZDs absorb UV light and hence can be excited efficiently by UV light. The analysis of BZDs by MALDI-TOF-MS was reported only on 7-aminoflunitrazepam (7-AF) [14] but its sensitivity was insufficient. That is, the limit of detection (LOD) was 2.5 μg/ml when 2,5-dihydroxybenzoic acid (DHB) was used as the matrix, and it was 0.25 μg/ml when DHB conjugated magnetic nanoparticles (DHB@MNP) was used as the matrix, although the LOD in urine was 30 ng/ml after 25 times concentration of 5 ml of urine. Therefore, in the present work we examined the applicability of MALDI-MS to the sensitive determination of the top 8 BZDs [1] with two of their metabolites using another matrix, α-cyano-4-hydroxy cinnamic acid (CHCA), in another solvent component.

The purifications of BZDs with liquid–liquid extraction (LLE) [2,4–6,8,9] and solid phase extraction (SPE) [3,7] were performed to eliminate large amounts of interfering substances contained in biological samples. Comparing the results on BZDs after LLE from serum [6] with those after SPE from plasma [7], they were equivalent in terms of recovery, linearity and accuracy. In the present work LLE was adopted for the purification of BZDs in blood because of its rapidness, low cost and applicability to a small amount of specimen. Multi-analytes identification as well as quantification

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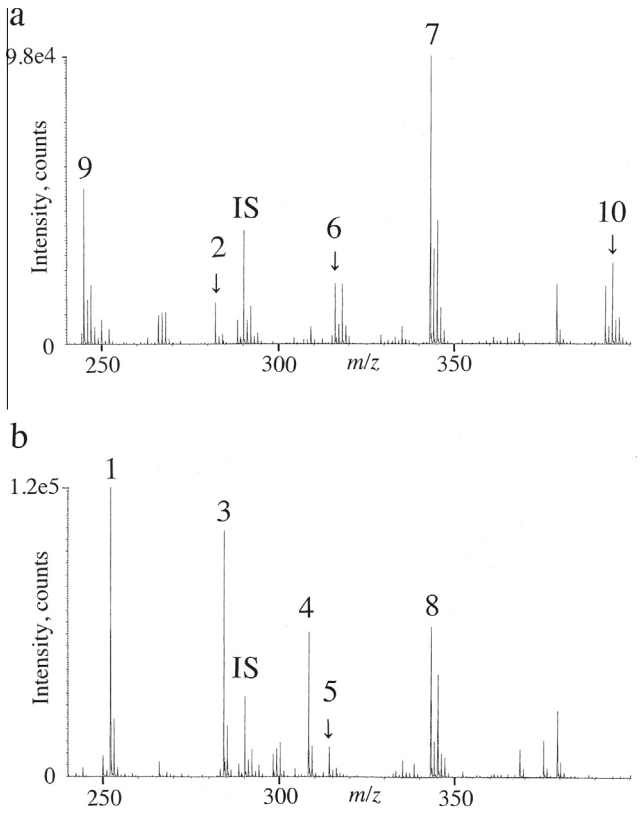

Fig. 1. MALDI-MS spectrum of 5 BZDs and IS at 200 ng/ml each (a), and that of the other 4 BZDs and IS at 200 ng/ml each as well as zolpidem at 20 ng/ml (b). The peak numbers from 1 to 10 correspond to 7-aminonitrazepam, nitrazepam, 7-aminoflunitrazepam, zolpidem, flunitrazepam, bromazepam, etizolam, triazolam, zopiclone and brotizolam, respectively.

of drugs in a single sample extract contributes to the saving of time and resources in forensic and clinical toxicology, and hence simultaneous detection of 8 BZDs was tried. The nitro-reduction metabolites of nitrazepam and flunitrazepam, i.e., 7-aminonitrazepam and 7-AF, respectively, were sometimes detected more frequently than the parent compounds in poisonous cases [2,3,7], and hence these two metabolites were also studied together in the present work. Unfortunately, simultaneous quantification of etizolam and triazolam could not be done because the *m/z* values of their protonated molecules, 343.0778 and 343.0511, respectively, were not separated enough for their quantification, but their identification could be done using either their exact masses in MALDI-TOF-MS or their product ion spectra in MALDI-MS/MS.

2. Materials and methods

2.1. Materials

7-AF was obtained from Hoffman-La Roche, Basel, Switzerland; 7-aminonitrazepam and diazepam-d_5 from Cerilliant, Round Rock, TX, USA; bromazepam and flunitrazepam from Eisai, Tokyo, Japan; brotizolam from Dainippon Pharmaceutical, Osaka, Japan; etizolam from Yoshitomi Pharmaceutical, Osaka, Japan; nitrazepam from Shionogi, Osaka, Japan; diazepam from Yamanouchi Pharmaceutical, Tokyo, Japan; triazolam, α-cyanono-4-hydroxy cinnamic acid (CHCA) and trifluoroacetic acid from Sigma–Aldrich, St. Louis, MO, USA; and DHB that was suitable for proteome research, acetonitrile (ACN) that was suitable for LC–MS, zopiclone and other chemicals of analytical grade from Wako Pure Chemicals, Osaka, Japan. Pure water with a specific resistance of 18 MΩ cm was used (Millipore, Bedford, MA, USA). Blood samples from healthy subjects under their permission with informed consent were used as blank samples, and those spiked with several amounts of BZDs were used as quality control samples. Blood, urine and gastric fluid of two victims were obtained at the autopsies performed in our laboratory, and the drug examination of these samples was asked officially.

2.2. Standard solutions

Individual stock solutions of BZDs were prepared separately by dissolving appropriate amounts of each drug in ethanol at 0.5 mg/ml and stored at −20 °C, respectively. Working calibration solutions and quality control solutions were prepared daily by dilutions of the stock solutions with blank blood at 0.7–200 ng/ml (one order lower in case of zolpidem). Diazepam-d_5 was used as internal standard (IS) at 200 ng/ml in blood.

Table 1
Data on MALDI-MS and MS/MS spectra of benzodiazepines. The compounds were numbered in the increasing order of molecular weight. Table indicated the name of compound with its molecular weight, count of the highest ion at 200 ng/ml with count of its blank, *m/z* values of main three ions in MS with relative intensities, and *m/z* values of main three product ions in MS/MS at collision of 40 V with relative intensities where the highest ion in MS was used for the precursor ion, respectively.

Compounds M.W.	Peak count Blank count	MS		MS/MS at 40 V
1. 7-aminonitrazepam	120,000	*m/z*	252, 253, 224	121, 94, 146
251.28	310	%	100, 17, 2	100, 11, 8
2. Nitrazepam	14,000	*m/z*	282, 267, 236	236, 180, 207
281.26	70	%	100, 63, 21	100, 68, 57
3. 7-aminoflunitrazepam	90,000	*m/z*	284, 285, 256	135, 226, 148
283.30	50	%	100, 18, 3	100, 26, 17
4. Zolpidem	540,000	*m/z*	308, 309, 235	235, 263, 221
307.39	70	%	100, 18, 3	100, 24, 6
5. Flunitrazepam	12,000	*m/z*	314, 299, 268	268, 239, 211
313.28	60	%	100, 58, 23	100, 41, 16
6. Bromazepam	20,000	*m/z*	316, 318, 317	182, 209, 260
316.16	30	%	100, 95, 26	100, 49, 12
7. Etizolam	98,000	*m/z*	343, 345, 344	314, 289, 259
342.85	280	%	100, 49, 34	100, 24, 21
8. Triazolam	64,000	*m/z*	343, 345, 344	308, 315, 239
343.22	80	%	100, 67, 22	100, 57, 28
9. Zopiclone	52,000	*m/z*	245, 101, 247	112, 139, 130
388.82	20	%	100, 47, 46	100, 21, 17
10. Brotizolam	28,000	*m/z*	395, 393, 397	314, 279, 341
393.70	80	%	100, 72, 27	100, 40, 23