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Determination of Midazolam in Animal Tissues by Solid-Phase Extraction HPLC-MS/MS Method

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Midazolam is usually used as ataractics for human. Addition of midazolam in animal feeds is forbidden by China Government for the health concern, however the residues of midazolam and its metabolites in animals can be inevitable found in food products that may cause drug resistance even have threaten to human health. On such basis, a reliable and sensitive method has been established and successfully applied for its determination in animal tissues by high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) analysis. Limit of detection of midazolam was 0.15 µg/kg and limit of quantification was 0.5 µg/kg. Midazolam was extracted from tissues samples with acetonitrile. The extract was purified by C18 solid phase extraction (SPE). The chromatographic separation was carried out on a C18 analytical column with a mobile phase composed of acetonitrile and 0.1 % formic acid. The presented method is sensitive and reproducible and thus suitable for accurate quantification of midazolam residue in animal-derived food products.

Keywords: Midazolam, Residue, Animal Tissues, HPLC-MS/MS.

INTRODUCTION

Midazolam ($C_{18}H_{13}N_3ClF$) is a short-acting benzodiazepine. Midazolam exerts a depressant action on the central nervous system and shares the sedative-hypnotic actions[1]. It can be used to alleviation or eliminate the animal manic state and keep it quiet[2]. The addition of midazolam in feeds can limit the animal movement and reduce the nutritional consumption. Thus, midazolam may be illegally added to animal feed to increase the body weight gain. However, the residues of midazolam and its metabolites in animal can be inevitable found in food products that cause drug resistance even endanger human body health badly. Notably, the metabities of midazolam in animal boby are mainly in livers and kidneys[3-9]. Further, the addition of midazolam in animal feeds is forbidden by China Government for the health concern[8]. It is very important to develop an effective method for the detection of midazolam in animal tissues. However, there is not an effective method to analysis midazolam in animal tissues.

Several chromatographic methods have been reported for determination of midazolam in plasma, serum and urine[9-15]. HPLC-UV methods have been published, but the limit of quantification for all these procedures is too high to be suitable[16-19]. Gas chromatographic (GC) method appears more sensitive than HPLC-UV. However, derivation is required by this method before analysis[20]. As a result, there is an urgent need to establish an effective method for the detection of midazolam in animal tissues. Unfortunately, no method has been reported so far about the detection of midazolam in feeds by high performance liquid chromatography coupled with tandem mass spectrometry.

In the present paper, a novel sensitive analytical method is established for the detection of midazolam in livers using liquid chromatography coupled with Tandem mass spectrometry. The method is successfully applied for the detection of midazolam residue in animal-derived food products.

EXPERIMENTAL

Midazolam standard (purity ≥ 99 %) dissolved in methanol was purchased from J&K Scientific Company (Beijing, China). The concentration of the standard solution was 1 mg/mL. Acetonitrile and methanol of HPLC grade were obtained from Merck (Darmstadt, Germany). Ultra pure water was produced by a Milli-Q water system (Millipore, Bedford, MA, USA). Other reagents were all of analytical grade and purchased from Beijing Fine Chemical Company (Beijing, China). Unless

otherwise indicated, all aqueous solutions were prepared with double distilled water. Purification cartridges (C18, 3 mL/200 mg) were purchased from waters (Made in Ireland). All sample solutions for HPLC analysis were filtered through a 0.22 μm polytetrafluoroethylene (PTFE) membrane filter purchased from Jingteng (Tianjin, China).

Preparation of working solution: The working solutions were prepared in methanol by diluting standard solution. Individual standard working solution was prepared by diluting standard solution. The concentration of working standard solutions were 0.5, 1, 5, 10, 50, 100 ng/mL. The working solutions were kept at 4 °C for further use.

Sample preparation and clean-up: Samples of liver from pig, cattle and chicken, respectively, were purchased from the supermarket or wholesale market of Beijing in March 2013 and stored at -20 °C in zip-lock plastic bags before analysis. Moreover, animal samples from organic certified farms were taken as blank matrix or for fortification. All samples were cut into thin slices after thawing for about 0.5 h and then homogenized by an Ultra Turrax (IKA, Staufen, Germany). An amount of 1 g of well-homogenized sample was taken into a 50 mL centrifuge tube and then was extracted with 10 mL acetonitrile by ultrasonic extraction for 15 min. After extraction, the solution was centrifuged at 12000 rpm for 15 min and the supernatant was collected. The solid residue was extracted for another time as above. Both supernatants collected were combined. Subsequently, the supernatant was evaporated to dryness at 40 °C in nitrogen atmosphere. The solid residue obtained was dissolved in 1 mL methanol. And 2 mL water was added to dilute the sample solution.

Samples were further purified using solid-phase extraction (SPE) on a C18 Cartridge. The solvent volume for each step of solid phase extraction was optimized. The cartridges were initially preconditioned by 3 mL methanol and 3 mL water, before the solution was loaded at a speed of 1 mL/min. Subsequently, the cartridges were washed by 3 mL water, dried for 1 min and eluted twice with 1 mL methanol at a speed of 1 mL/min. The eluant was evaporated to dryness at 40 °C in nitrogen atmosphere. The solid residue obtained was dissolved in 1 mL 50 % acetonitrile solution and analyzed using HPLC.

HPLC-tandem mass spectrometer and conditions: Analysis of midazolam was carried out on an Agilent 1200 liquid chromatography system (equipped with a binary pump, vacuum degasser, autosampler, Palo Alto, CA, USA) coupled with API 5000 mass spectrometer with electrospray ionization (ESI). A Waters XTerra® MS C18 column (150 mm × 2.1 mm id, 5 μm) was adopted with a guard column (2.1 × 10 mm, 3.5 μm, Waters, Made in Ireland). The mobile phase consisted of 0.1 % formic acid in water (solvent A) and acetonitrile (solvent B). All HPLC analysis was performed under a gradient elution: 0-5 min, 80-40 % A; 5-5.5 min, 40-5 % A; 5.5-10 min, 5-5% A; 10-10.1 min, 5-80 % A; 10.1-15 min, 80-80 % A, at a flow rate of 0.3 mL/min. An aliquot of each sample solution (5 μL) was injected into the HPLC system for analysis.

Tandem mass spectrometry used was an API 5000 Triple Quadrupole from Applied Biosystem (ON, Canada). The analyte was ionized in a positive ionization mode with multiple reaction monitoring (MRM) at ion spray voltage of 5500 v and ion source temperature of 500 °C. The flow rate of curtain gas is 35 psi and 60 psi for both ion source gas 1 and 2, respectively. All gases were supplied with nitrogen (purity > 99.995 %). The parameters mentioned above were optimized by flow injection analysis (FIA) program using a midazolam solution at concentration of 10 μg/kg.

For the method establishment, scan mode on Q1 analyze was performed for precursor ion in the range (*m/z*) from 50 to 400. The *m/z* of 326.1 was identified as midazolam precursor ion. Product ions were indentified in Q2. Representative product ions were selected as listed in Table-1. Declustering potentials (DP) and collision energies (CE) were optimized for each MRM transition using the ramp function.

TABLE-1
OPTIMIZATION INDEXES OF MASS SPECTROMETRY FOR MIDAZOLAM

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	DTc (ms)	DPd (V)	CEe (V)
Midazolam	326.1	291.1a	150	51	45
		249.1b	150	66	55
		223.1b	150	66	57

[a]Ion for quantification, [b]Ion for confirmation, [c]Dwell time, [d]declustering potential, [e]collision energy

Validation of the method: Method validation was performed in terms of accuracy, precision, linearity and detectability. Accuracy is expressed as the closeness of the standard samples to the actual known amount. The accuracy was evaluated as percentage recovery, *i.e.*, (concentration found/concentration added) × 100 %. The accuracy was tested on the validation samples at concentrations of 0.5, 1 and 5 μg/kg.

Precision is the level of repeatability as reported between samples analyzed on the same day (intraday) and on three different days (inter-day). It was assessed by the coefficient of variation (CV). Intra-day precision was tested based on the validation samples at concentrations of 0.5, 1 and 5 μg/kg analyzed on the same day, while the inter-day precision was determined on three different working days.

The linearity was evaluated by adding midazolam standards at concentrations of 0, 0.5, 1, 5, 10, 50 and 100 μg/kg to each of three kinds of blank feed sample extraction solutions. Each sample was analyzed with triplicate injections. The standard curves were calculated using linear regression of the peak area. Linearity of the analysis was determined from the correlation coefficients (r) obtained using linear regression analysis between the concentration *versus* the peak area for midazolam.

The detectability of the method was demonstrated by the limit of detection (LOD) and the limit of quantification (LOQ) for midazolam. Both LOD and LOQ were estimated based on a signal-to-noise ratio of 3 and 10, respectively.

RESULTS AND DISCUSSION

Sample preparation and clean-up: It is difficult, but critical for the detection, to extract midazolam from the samples of liver with as low interference and as high recovery as possible. In order to establish a reliable extraction procedure, the conditions were optimized, including: extraction solvents (acetonitrile, methanol, ethyl acetate, *n*-hexane, acetone and ethanol), extraction durations (10, 15 and 20 min) and the extraction methods (vertical shaking and ultrasonic extraction).

It has been suggested that too many impurity compounds were extracted when using *n*-hexane, acetone and ethanol as the solvent. Although the extraction by methanol led to fewer impurities, the recovery rate was extremely low. Finally, acetonitrile was chosen as the extraction solvent since both higher recovery rate and fewer impurity compounds can be achieved as compared with other extraction solvents. No significant difference was observed between ultrasonic extraction and vertical shaking for 15 min extraction. The recovery rate increased little when the extraction time was further enhanced to 20 min.

It is still not suitable for high performance liquid chromatography coupled with tandem mass spectrometry direct injection using the extraction solution and thus, sample purification has been optimized by solid-phase extraction (SPE), which can effectively reduce matrix effects and ion suppression for analytical assays. Solid phase extraction cartridges were compared, including C18, SI and HLB solid phase extraction Cartridges with a variety of loading volumes (100, 200 and 500 mg). It has been indicated by the results that impurity compounds can be removed by both C18 and SI SEP cartridge, but the recovery rate was less than 70 % for the latter one. Based on the results mentioned above, C18 solid phase extraction cartridge is selected for the clean-up of the extraction. The optimum loading volume has also been determined at 200 mg, since it will easily break through for the volume of 100 mg and difficult for elution at a volume of 500 mg.

LC-MS/MS: For the analysis of midazolam by mass spectrometry, both ESI and atmospheric pressure chemical ionization (APCI) sources were compared, in which ESI gave better sensitivity than APCI. To enhance the positive ionization condition, 0.1 % formic acid in water was used in the mobile phase. Gradient flow was optimized to give better resolution. As shown in Fig. 1, the product ion of *m/z* 291.1 was chosen as the quantitative analysis ion. Representative chromatograms of three feed blank solutions and spiked samples were shown in Fig. 2 a, c, e and 2 b, d, f, respectively.

Method validation: Method validation was carried out on three kinds of livers. Analysis of blank feed samples was also included in order to verify the absence of the target analyte and potential interfering compounds. As shown in Table-2, the average recoveries for all there types of liver were higher than 71 %. The intra-day and inter-day coefficients of variation were less than 8 % (Table-3).

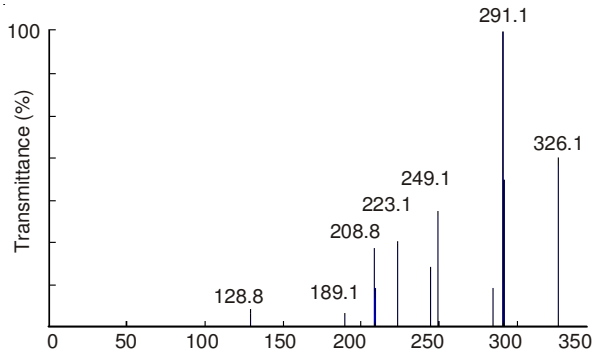

Fig. 1. MS spectra of product ions of midazolam

TABLE-2
RECOVERIES FOR MIDAZOLAM IN VALIDATION SAMPLES

Analyte	Spiked level (µg/kg)	Recovery (mean ± RSD %, n = 3)		
		Pig liver	Cattle liver	Chicken liver
Midazolam	0.5	71.44 ± 7.48	73.45 ± 6.97	71.53 ± 4.58
	1	74.13 ± 7.67	72.99 ± 6.43	72.48 ± 7.47
	5	77.12 ± 5.18	79.34 ± 3.79	76.74 ± 6.51

The linear equations, correlation coefficients (r) and linear range of midazolam were listed in Table-3. Only slight variations were observed among the slopes calculated on different days and good linearities were found within all tested intervals. The detectability was demonstrated by the limit of detection (LOD) and the limit of quantification (LOQ). Both LOD and LOQ were estimated based on a signal-to-noise ratio of 3 and 10, respectively, as listed in Table-4. The results indicated the suitability of the proposed method for the determination of trace concentration of midazolam. It has been demonstrated by the results that the established method in this study is better than other methods using HPLC-UV [4,13,14].

Conclusion

In this study a high sensitive LC-MS/MS method for simultaneous determination of midazolam residue in animal tissues was developed. The method shows good recovery at different spiking levels tested and a satisfying performance particularly in terms of selectivity and sensitivity also thanks to the high selectivity offered by the MS/MS approach. Compared to other methods reported a lower LOD was achieved in the investigated matrices with an improved clean-up step employed. The method demonstrated also suitable for midazolam residue surveillance in animal-derived food.

TABLE-3
INTRA-DAY AND INTER-DAY PRECISION FOR MIDAZOLAM IN VALIDATION SAMPLES

Analyte	Spiked level (µg/kg)	Intra-day coefficient of variation (CV, %)			Analyte	Spiked level (µg/kg)	Inter-day coefficient of variation (CV, %)		
		Pig liver	Cattle liver	Chicken liver			Pig liver	Cattle liver	Chicken liver
Midazolam	0.5	3.62	3.93	4.21	Midazolam	0.5	3.69	4.71	3.74
	1	3.46	3.98	3.07		1	2.58	3.91	3.87
	5	2.53	3.16	4.02		5	3.56	3.12	4.13

TABLE-4
LINEARITY AND DETECTABILITY OF MIDAZOLAM

Analyte	Regression equation	Linear range (µg/kg)	Correlation coefficient (r)	LOD (µg/kg)	LOQ (µg/kg)
Midazolam	y = 615.59 x-352.38	1-100	0.9991	0.3	1.0

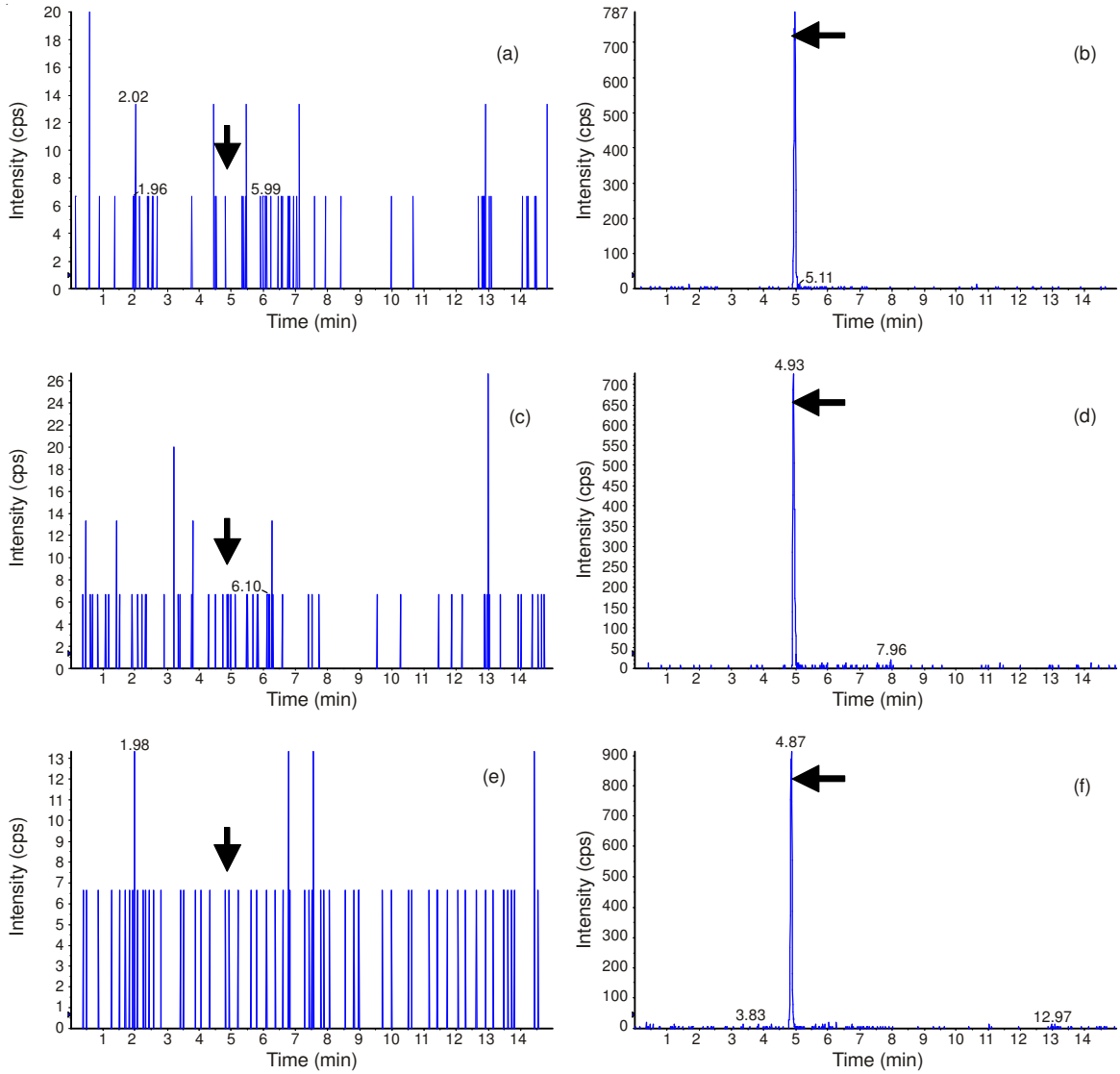

Fig. 2. MRM chromatograms of samples from pig feeds without (a, c, e) or with (b, d, f) midazolam (5 µg/kg). (a, b) pig liver. (c, d) cattle liver. (e, f) chicken liver. All of the chromatograms were the product ion *m/z* 291.1

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REFERENCES

1. J.G. Reves, R.J. Fragen, H.R. Vinik and D.J. Greenblatt, *Anesthesiology*, **62**, 310 (1985).
2. L. Quintieri, P. Palatini, A. Nassi, P. Ruzza and M. Floreani, *Biochem. Pharmacol.*, **75**, 1426 (2008).
3. J.C. Gorski, S.D. Hall, D.R. Jones and M. Vanden Branden, *Biochem. Pharmacol.*, **47**, 1643 (1994).
4. T. Sano, K. Sato, R. Kurihara, Y. Mizuno, T. Kojima, Y. Yamakawa, T. Yamada, A. Ishii and Y. Katsumata, *Leg. Med.*, **3**, 149 (2001).
5. R. Vrzal, K. Kubesova, P. Pavek and Z. Dvorak, *Toxicol. Lett.*, **193**, 183 (2010).
6. F. Elbarbry, A. Attia and A. Shoker, *J. Pharm. Biomed. Anal.*, **50**, 987 (2009).
7. M. Shimizu, T. Uno, H.- Tamura, H. Kanazawa, I. Murakami, K. Sugawara and T. Tateishi, *J. Chromatogr. B*, **847**, 275 (2007).
8. Ministry of Agriculture of the People's Republic of China, Available online: http://www.moa.gov.cn/zwllm/tzgg/gg/201104/t20110422_1976307.htm.
9. F. Rubio, B.J. Miwa and W.A. Garland, *J. Chromatogr. A*, **233**, 157 (1982).
10. J.X. de Vries, J. Rudi, I. Walter-Sack and R. Conradi, *Biomed. Chromatogr.*, **4**, 28 (1990).
11. H. Kanazawa, R. Nishimura, N. Sasaki, A. Takeuchi, N. Takai, Y. Nagata and Y. Matsushima, *Biomed. Chromatogr.*, **9**, 188 (1995).
12. M.R. Shiran, A. Gregory, A. Rostami-Hodjegan, G.T. Tucker and M.S. Lennard, *J. Chromatogr. B*, **783**, 303 (2003).
13. S.A. Testino Jr. and G. Patonay, *J. Pharm. Biomed. Anal.*, **30**, 1459 (2003).
14. A. El Mahjoub and C. Staub, *J. Chromatogr. B Biomed. Sci. Appl.*, **742**, 381 (2000).
15. K.M. van Rij, D. Compas, E.L. Swart, P.N. de Goede and D.J. Touw, *Ther. Drug Monit.*, **21**, 416 (1999)
16. N. Yasui-Furukori, Y. Inoue and T. Tateishi, *J. Chromatogr. B*, **789**, 239 (2003).
17. E.J. Portier, K. de Blok, J.J. Butter and C.J. van Boxtel, *J. Chromatogr. B*, **723**, 313 (1999).
18. P.G. ter Horst, N.A. Foudraine, G. Cuypers, E.A. van Dijk and N.J. Oldenhof, *J. Chromatogr. A*, **791**, 389 (2003).
19. Q. Jiang, N.Y. Walton, S. Gunawan and D.M. Treiman, *J. Chromatogr. B*, **683**, 276 (1996).
20. F. Rubio, B.J. Miwa and W.A. Garland, *J. Chromatogr. A*, **233**, 167 (1982).