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Development and Validation of LC/MS/MS Method with Ultra Small-Volume Injection for Quantitative Determination of Alprazolam in Human Plasma

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1. Introduction

The triple quadrupole LC/MS/MS has been used widely in drug discovery, bioequivalence and clinical applications for high throughput quantitative analysis of drug molecules and metabolites in biological samples. Due to the excellent mass selectivity, quantitative analysis by MRM technique could be carried out with minimum LC separation of the targets from the biological matrix components, achieving very fast analysis speed. The further improvement in sensitivity and robustness of new generation triple quadrupole LC/MS/MS in recent years allows the use of simplified procedure in pre-treatment of plasma samples (e.g., protein precipitation). However, ion suppression and contamination of the interface and ion optics by dirty biological matrix are often the causes of decreased sensitivity and poor repeatability. One of the methods is to reduce the loading amount of biological samples onto the LC/MS/MS system, but it may sacrifice the sensitivity and requires more reliable Autosampler to ensure no compromise in sample carryover, accuracy and epeatability. We report here a MRM method for quantitative determination of alprazolam (a psychoactive drug) in human plasma with an ultra small-volume injection (0.1uL) on the LCMS-8080 coupled with LC-30A UHPLC system. The performance of the method was evaluated. This study reveals that the ultra small-volume (0.1 uL) injection method was proven to be well performed in comparison with the normal method (5~10 uL). The advantage of the method was not only minimizing the contamination of sample matrix to the interface and ion optical lens, but also reducing the amount of biological samples needed.

2. Experimental

A simple sample treatment method was applied: to 50 uL of blank human plasma spiked with a desired amount of alprazolam ($C_{17}H_{13}ClN_4$) stock solution and topped up to 150 uL with acetonitrile, followed by vortexing for 1 minute. The spiked sample was then centrifuged at 12K rpm for 10 minutes. The supernatant was filtered (0.2 mu filter) into a 100 uL insert sample vial.
The sample was injected onto LC/MS/MS for quantitative determination of the drug. For method development and performance evaluation, a series of spiked plasma samples and standard solutions in mobile phase (50:50 in vol) were prepared. A LCMS-8080 triple quadruple coupling with a Nexera UHPLC (Shimadzu Corporation, Japan) was used in this study. The details of the analytical conditions are shown in Table 1. A SIL-30AC Autosampler as a standard module of the Nexera system was used for injection of an ultra small-volume (0.1 uL) of plasma samples.
A standard washing procedure (pre- and after-injection mode) of the needle and injection port with MeOH/water (50:50 in vol) as the washing solvent was selected and used in this study.

3. Results and Discussion

3.1 Establishment of quantitative method with ultra small-volume injection

The MRM parameters of alprazolam was optimized on LCMS-8080 and two transitions were used, with 309.1>281.1 for quantification and 309.1>274.1 for confirmation. As shown in Fig. 1, the alprazolam peak appeared at 3.2 min and the total run time was 4.5 min including column washing and re-equilibrium.
Quantitative calibration curves were established based on 309.1> 281.1 MRM transition using the spiked plasma samples as described above. The calibration curve with an injection volume of 10 uL is shown in Fig. 2. Excellent linearity ($R^2 > 0.9981$) was obtained for a wide concentration range from 4.67 ppt to 13,333 ppt, which correspond to the on-column loading amounts from 46.7 fg to 133.3 pg.

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Table 1 LCMSMS system and analytical conditions

Instrument:	LCMS-8080 with Nexera UHPLC
Mobile Phase A:	Water with 0.1% FA
Mobile phase B:	MeOH
Total flow rate:	0.4 ml/min
Column:	FC-ODS, 3 um, 150 x 3 mm
Gradient Elution B:	0~0.2 min, 40%→ 2.5~3.5 mn, 90% →3.51~4.5 min, 40%
Injection vol:	0.1 or 10 uL
Interface:	ESI with HSID
Probe temp:	450°C
HSID temp:	300°C
Nebulizer Gas:	2.0 L/min(Purified Air)
Heating gas:	12.0 L/min (Purified Air)
Curtain gas:	2.7 L/min (N2 generator)
Collision gas:	4.0 (L/min) (N2 generator)
Exhaust gas:	on (Purified Air)

Table 2 MRM parameters of Alprazolam on LCMS-8080

MRM transition	Dwell time (ms)	EV (V)	CE (V)	CCL4 (V)
309.1 > 281.1	50	40	-34	-8
309.1 > 274.1	50	50	-34	-8

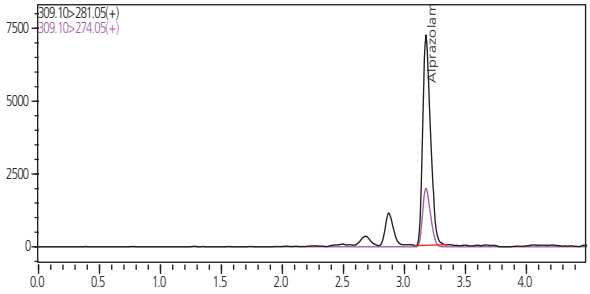

Fig. 1 MRM chromatogram of alprazolam in plasma (0.233 ppb × 10 uL).

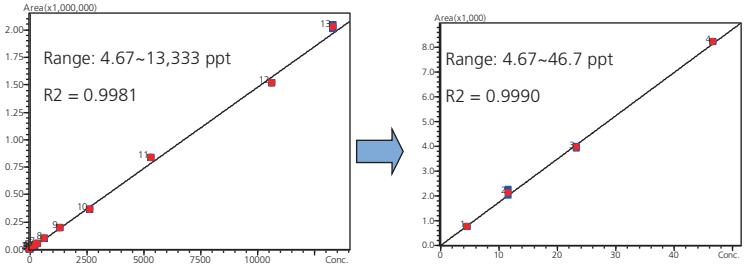

Fig. 2 Calibration curve of alprazolam in plasma for a wide dynamic range from 4.67 ppt to 13,333 ppt (injection volume = 10 uL) on LCMS-8080.

Fig. 3 shows the calibration curve established by employing an ultra small-volume injection, i.e., 0.1 uL for concentrations from 333 ppt to 10,666 ppt, which corresponds to 33.3 fg to 1066.7 fg on column. The linearity (R2 > 0.999) of the ultra small-volume injection method was as good as that of 10uL injection method (Fig. 2).

3.2 Validation and Quantification of Screening Results

The repeatability, accuracy and LOD/LOQ were evaluated at two levels of spiked plasma samples: 66.7 and 266.7 fg on column with 0.1 uL injection. The results are shown in Fig. 4 and Tables 3(a) and (b). The LOD and LOQ of the method were 14.5 fg (S/N =3) and 43.5 fg (S/N= 10), respectively. The MRM chromatogram of the lowest calibration point (33.3 fg), which is more closed to the LOQ of the method, is shown in Fig. 4.

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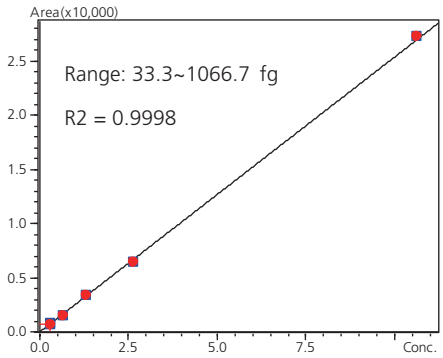

Level	Conc. (pg/uL)	Inj Vol (uL)	Amount (fg)
1	0.333	0.1	33.3
2	0.667	0.1	66.7
3	1.333	0.1	133.3
4	2.666	0.1	266.6
5	10.666	0.1	1066.6

Fig. 3 Calibration curve established with ultra small injection volume (0.1 uL) of alprazpam in plasma on LCMS-8080

The repeatability (RSD %, n=8) of peak areas for 66.7 fg and 266.7 fg on column were 8.7% and 2.9%, espectively. The concentration accuracy for the two levels was at 79~101% and 108~117%, respectively. The above performance data were compared with that of injection volume of 10 uL and the results showed no significant differences. The excellent repeatability and good accuracy obtained with 0.1 uL injection was attributed toboth of stability of the MS system and the declared features of near-zero sample carryover and highly precise injection of the SIL-30A autosampler by Shimadzu [1]. It is worth to note that sample carryover by injection needle and/or injection port of an autosampler is a key potential problem in such ultra small-volume injection method, because even a very small amount of residual sample remaining on the needle or injection port could cause a significant contribution to the next injection. The above result could be regarded as an indirect evidence of the "near zero sample carryover" feature which was due to the special design and surface treatment of the needle and injection port [1].

Table 3 (a) Repeatability of alprazolam peak of spiked plasma sample with 0.1 uL injection Volume. Loading amount: 66.7 fg on-column

Inj No.	Data Filename	Ret. Time	Peak Area	Std. Conc.	Conc. (pg/uL)	Accuracy [%]	S/N	Detect. Limit (DL)	Quant. Limit (QL)
1	CSC 711.lcd	3.16	1,336	0.667	0.526	78.9	11.99	0.14	0.44
2	CSC 712.lcd	3.16	1,485	0.667	0.585	87.7	13.16	0.15	0.44
3	CSC 713.lcd	3.16	1,451	0.667	0.571	85.6	10.95	0.17	0.52
4	CSC 714.lcd	3.16	1,482	0.667	0.584	87.6	15.32	0.13	0.38
5	CSC 715.lcd	3.16	1,710	0.667	0.674	101.0	18.65	0.12	0.36
6	CSC 716.lcd	3.16	1,368	0.667	0.539	80.8	13.16	0.14	0.41
7	CSC 717.lcd	3.16	1,436	0.667	0.566	84.9	16.00	0.12	0.35
8	CSC 718.lcd	3.16	1,645	0.667	0.648	97.2	14.62	0.15	0.44
	Average	3.16	1,489		0.587		14.23	0.14	0.42
	%RSD	0.04	8.63		8.65		17.22	13.17	13.17

Table 3 (b) Repeatability of alprazolam peak of spiked plasma sample with 0.1 uL injection Volume. Loading amount: 266.6 fg on-column

Inj No.	Data Filename	Ret. Time	Area	Std. Conc.	Conc. (pg/uL)	Accuracy [%]	S/N	Detect. Limit(DL)	Quant. Limit(QL)
1	CSC 719.lcd	3.157	6,321	2.666	2.918	109.5	43.28	0.22	0.67
2	CSC 720.lcd	3.163	6,651	2.666	3.07	115.2	62.28	0.16	0.49
3	CSC 721.lcd	3.156	6,648	2.666	3.069	115.1	64.36	0.16	0.48
4	CSC 722.lcd	3.158	6,278	2.666	2.898	108.7	78.63	0.12	0.37
5	CSC 723.lcd	3.158	6,598	2.666	3.046	114.3	81.5	0.12	0.37
6	CSC 724.lcd	3.157	6,805	2.666	3.141	117.8	96.54	0.11	0.33
7	CSC 725.lcd	3.159	6,575	2.666	3.035	113.8	67.21	0.15	0.45
8	CSC 726.lcd	3.159	6,763	2.666	3.122	117.1	69.46	0.15	0.45
	Average	3.16	6580		3.04		70.41	0.15	0.45
	%RSD	0.06	2.89		2.88		22.29	23.79	23.79

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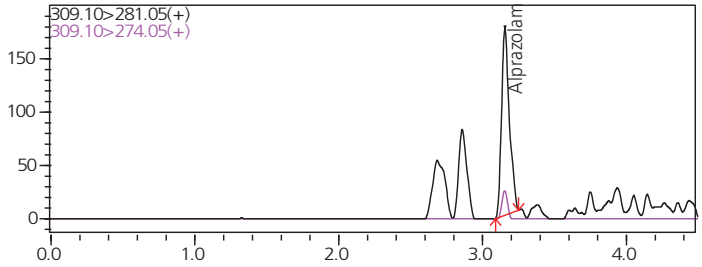

Fig. 4 MRM chromatogram of alprazolam in plasma, 33.3 fg on column (0.333 ppb x 0.1 uL), S/N = 8.67 (Noise: 3.5~4 min)

In addition, matrix effect of the method was also investigated. The results (see Table 4) indicate that the injection volume did not change the matrix effect behaviour. However, more significant matrix effect occurred in low concentration samples due to ion suppression effect.

Table 4 Matrix effect at different injection volume and loading amount

Conc. (ng/ml)	Inj vol (uL)	On-column (fg)	Peak Area		Matrix Effect (%)
			in solvent	in plasma	
0.233	0.1	23.3	290	194	66.9
0.233	5	1165	30971	15350	49.6
0.233	10	2330	55275	32506	58.8
13.33	0.1	1333	16541	20400	123.3
13.33	5	66650	1197786	1078876	90.1
13.33	10	133300	2262305	2019131	89.3

3.3 Reduced contamination by ultra small-volume injection method

Chemical contamination of ESI interface and ion inlet/optics may cause decrease of electrical conductivity and discharge on the surface of the devices, which are often the cause of decrease of ionization efficiency of the target compounds. There are various solutions to reduce chemical contamination of LC/MS/MS such as off-line purification by SPE or L-L extraction, on-line SPE etc. Reduction of sample loading amount is a most simple method without any investment and technical effort on the system or analytical method. However, the arguments of the ultra smallvolume injection method are first the sacrifice of sensitivity and second the actual effectiveness in reduction of contamination. It has been observed that in general the frequency of cleaning of ESI interface seems to be reduced. However, it is still lack of quantitative evidences and proof at this time. The sensitivity sacrifice could be adjusted by selecting more appropriate injection volumes of sub microlitres (0.1~1 uL) according to the required sensitivity of an analysis project.

4. Conclusions

A sensitive LC/MS/MS method with ultra small-volume injection for quantitative analysis of alprazolam in human plasma was set up and the performance was evaluated. The results of method performance evaluation indicate that it is possible to use 0.1 uL injection in quantitative determination of alprazolam in plasma samples. The purpose of using ultra small-volume injection was essentially to reduce of chemical contamination of ESI interface and ion inlet/optics by dirty biological samples, so as to prevent frequent cleaning and maintenance efforts. It is also to explore the possibilities and methods to analyze various dirty samples like whole bloods directly without sample pre-treatment.

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5. References

[1] LC world talk 2011 issue 1 (C190-E149), http://www.shimadzu.com/an/lc_worldtalk/index.html

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