Application Note 571

Simultaneous Quantitation of 19 Drugs in Human Plasma and Serum by LC-MS/MS

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Key Words

TSQ Vantage, drug monitoring research, clinical research, CSS, plasma, serum

Goal

To develop a simple, fast, and sensitive LC-MS/MS method for the simultaneous quantitation of 19 drugs in human plasma and serum.

Introduction

Liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) has become an accepted tool for quantitative analysis of drugs in clinical research laboratories. LC-MS/MS enables simultaneous, sensitive detection and quantitation of multiple analytes of interest. In this study, 19 drugs of various types, including antipsychotics, antiepileptics/anticonvulsants, antianginals, and antidepressants, were monitored and simultaneously quantitated using LC-MS/MS.

Experimental

Sample Preparation

Nineteen drugs (Table 1) and 15 isotopically labeled internal standards of the drugs were used in this research.

Table 1. Drug analytes

Analytes		
Amitriptyline	Dothiepin	Nortriptyline
Bromazepam	Doxepin	Oxazepam
Clobazam	Flunitrazepam	Perhexilline
Clomipramine	Imipramine	Temazepam
Clonazepam	Lamotrigine	Trimipramine
Clozapine	Levetiracetam	
Diazepam	Nitrazepam	

To assess signal recovery and determine the best dilution factor, 9 randomly chosen individual human-donor plasma samples were spiked with the 19 drugs at 40 ng/mL and 15 isotopically labeled internal standards at 100 ng/mL. These samples were mixed (1:3, v/v) with a 1:1 methanol/acetonitrile mixture. The samples were vigorously vortexed and stored at -30 °C for 30 min. The samples were then centrifuged at 17,000 *g* for 5 min. Supernatant (20 µL) was drawn off and diluted 10-fold, 20-fold, and 50-fold with 10% methanol in water to final dilution factors of 40x, 80x, and 200x.

Calibration and linearity standards were prepared by spiking a matrix of charcoal-stripped human serum (CSS) with the 15 internal standards at 100 ng/mL and the 19 drug analytes at 4, 10, 20, 40, 100, 200, and 400 ng/mL. The samples were processed as above and diluted to a final dilution factor of 200x.

For accuracy and precision testing, CSS samples were spiked with the 15 isotopically labeled internal standards at 100 ng/mL and the 19 drugs at both 40 ng/mL and 200 ng/mL. The samples were processed as above and diluted to a final dilution factor of 200x.

Also for accuracy and precision testing, 9 individual human-donor plasma samples were spiked with the 15 isotopically labeled internal standards at 100 ng/mL and the 19 drugs at 40 ng/mL. The samples were processed as above and diluted to a final dilution factor of 200x.

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Liquid Chromatography

Chromatographic separations were performed with a Thermo Scientific Accela 1250 pump and Accela Open autosampler. The analytical column was a Thermo Scientific Accucore PFP column (50 × 2.1 mm, 2.6 µm particle size). The column was maintained at room temperature. Details of the LC gradient and information on the mobile phases (MP) are shown in Table 2. The injection volume was 40 µL.

Table 2. LC gradient

Time (min)	Flow rate (mL/min)	Gradient	MPA (%)	MPB (%)	MPC (%)
0.00	0.4	Step	95	5	0
0.50	0.4	Step	90	10	0
1.50	0.4	Ramp	50	50	0
2.00	0.4	Ramp	5	95	0
6.50	0.4	Step	0	100	0
7.75	0.6	Step	0	0	100
8.00	0.6	Step	95	5	0

MPA: 10 mM ammonium acetate and 0.1% formic acid in water
MPB: 10 mM ammonium acetate and 0.1% formic acid in methanol
MPC: acetonitrile:isopropanol:acetone 9:9:2 (v/v/v)

Mass Spectrometry

MS/MS analysis was performed on a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer. The mass spectrometer was operated with a heated electrospray ionization (HESI-II) source in positive ionization mode. The MS conditions were as follows:

Spray voltage (V):	4000
Vaporizer temperature (°C):	300
Sheath gas pressure (arbitrary units)	50
Auxiliary gas pressure (arbitrary units)	15
Capillary temperature (°C)	300

Data were acquired in selected-reaction monitoring (SRM) mode. Detailed SRM settings for the 19 drugs and their internal standards are shown in Table 3. For each analyte and internal standard, two SRM transitions were monitored. One was used as the quantifier and the other as the qualifier. The signal ratio between the qualifier and the quantifier was used to evaluate the validity of the results. Results that varied by more than 20% of the nominal ratio were considered invalid data points.

The validation procedure included tests for: 1) signal recovery, 2) lower limit of quantitation (LLOQ) and linear range, 3) accuracy and precision, and 4) carryover.

Table 3. SRM settings for the analytes and internal standards

Analyte	Precursor Ion (*m/z*)	Quantifier Ion (*m/z*)	Collision Energy (V)	Qualifier Ion (*m/z*)	Collision Energy (V)	S-Lens (V)
Amitriptyline	278.10	202.10	56	233.10	16	74
Bromazepam	316.11	182.10	31	209.10	26	95
Clobazam	301.10	259.10	20	224.10	32	90
Clomipramine	315.10	86.00	17	58.00	35	74
Clonazepam	316.00	270.10	25	214.00	37	101
Clozapine	327.10	270.10	23	192.00	42	94
Diazepam	285.10	193.10	32	154.00	27	88
Dothiepin	296.10	202.10	53	221.10	45	71
Doxepin	280.10	165.10	51	107.00	23	80
Flunitrazepam	314.10	268.10	26	239.10	34	92
Imipramine	281.20	86.00	16	58.00	35	69
Lamotrigine	256.00	211.00	26	109.00	49	89
Levetiracetam	171.10	126.10	14	69.00	28	36
Nitrazepam	282.10	236.10	24	207.10	34	97
Nortriptyline	264.20	233.20	13	91.10	32	66
Oxazepam	287.10	269.10	14	104.10	33	81
Perhexilline	278.20	95.10	28	67.00	34	87
Temazepam	301.11	255.10	22	283.10	13	72
Trimipramine	295.20	100.10	16	58.10	35	71
Internal Standards						
Amitriptyline-D3	281.21	91.10	32	233.20	16	85
Clomipramine-D3	318.20	89.10	18	61.10	36	75
Clonazepam-D4	320.10	274.10	26	218.10	35	102
Clozapine-D4	331.20	272.20	25	192.10	45	102
Diazepam-D5	290.10	198.10	31	154.00	26	89
Doxepin-D3	283.20	107.00	23	77.00	46	78
Flunitrazepam-D7	321.10	275.20	26	246.20	35	96
Imipramine-D3	284.20	89.10	16	61.10	35	69
Lamotrigine-13C, 15N4	261.00	214.00	26	109.10	50	104
Levetiracetam-D6	177.10	132.20	14	69.10	30	38
Nitrazepam-D5	287.11	185.10	37	212.10	34	100
Nortriptyline-D3	267.20	91.00	33	233.20	14	66
Oxazepam-D5	292.10	246.10	22	274.10	15	84
Temazepam-D5	306.10	260.10	23	288.10	13	83
Trimipramine-D3	298.20	103.10	16	61.10	35	72

Results and Discussion

Signal Recovery

Plasma and serum are complex matrices. The matrix content in them can significantly affect the detection of drugs by ESI MS. Therefore, three different dilution factors after protein precipitation (40-fold, 80-fold, and 200-fold) were compared. The LC-MS/MS signals of the analytes in the plasma samples were compared to LC-MS/MS signals from solvent blanks with the same spikes. The 200-fold sample dilution produced the best signal recovery and minimum ion suppression (Table 4 and Figures 1 and 2). For all of the subsequent analyses, all samples were prepared with a 200-fold final dilution factor.

Table 4. Absolute mean signal recovery of 19 drugs at 40 ng/mL in 9 human plasma samples diluted 40-fold, 80-fold, and 200-fold, as compared to a similarly spiked solvent blank

Analyte (40 ng/mL)	Absolute mean signal recovery (%)		
	n=9 200x dilution	n=9 80x dilution	n=9 40x dilution
Amitriptyline	107.9	53.9	79.4
Bromazepam	125.7	49.7	56.6
Clobazam	78.6	43.4	54.4
Clomipramine	103.6	57.5	84.1
Clonazepam	65.9	36.0	32.3
Clozapine	81.5	60.4	56.7
Diazepam	78.4	45.6	57.9
Dothiepin	124.6	53.4	83.9
Doxepin	110.8	57.4	84.0
Flunitrazepam	77.8	44.1	51.9
Imipramine	107.2	50.6	82.8
Lamotrigine	71.5	45.1	52.8
Levetiracetam	86.7	48.2	58.3
Nitrazepam	77.8	38.4	41.7
Nortriptyline	83.7	44.5	62.2
Oxazepam	74.5	41.9	52.7
Perhexilline	94.9	152.8	190.0
Temazepam	74.7	44.6	55.1
Trimipramine	98.4	49.1	76.4

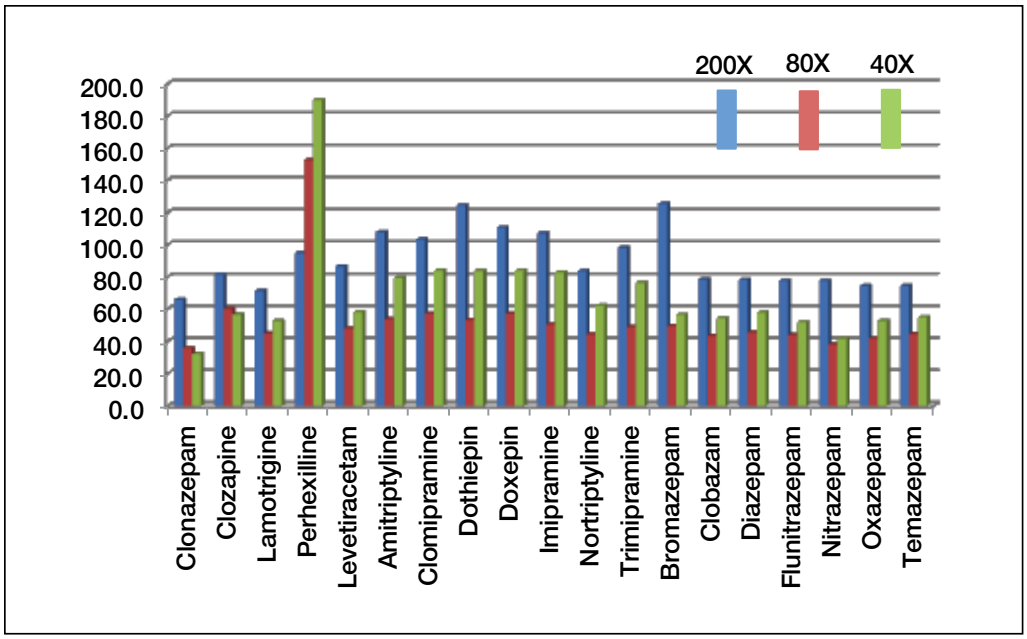

Figure 1. Mean signal recovery of 19 drugs at 40 ng/mL in 9 human plasma samples diluted 40-fold, 80-fold, and 200-fold, as compared to a similarly spiked solvent blank

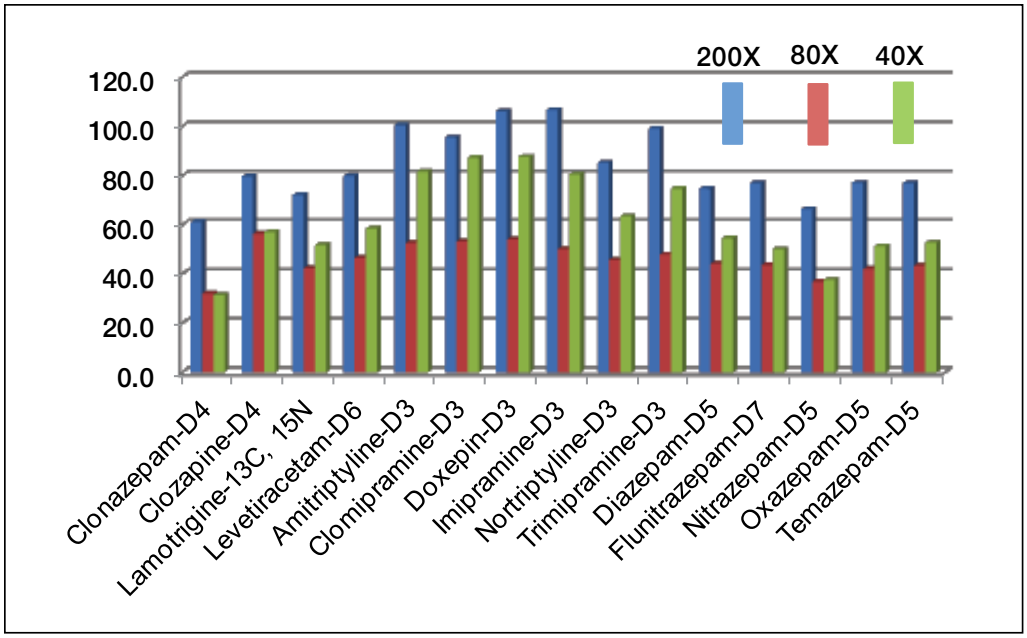

Figure 2. Mean signal recovery of 15 internal standards at 100 ng/mL in 9 human plasma samples diluted 40-fold, 80-fold, and 200-fold, as compared to a similarly spiked solvent blank

Lower Limit of Quantitation and Linear Range

The lower limit of quantitation (LLOQ), linearity, and ion ratio test parameters for the 19 drugs are summarized in Table 5. For calibration curves, a linear fit with 1/X weighting was used. The LLOQ for these 19 drugs were determined to be between 4 and 20 ng/mL. The method was linear to 400 ng/mL for all the drugs. Figure 3 shows the calibration curve of clozapine in CSS. Figure 4 shows the overlaid SRM chromatograms (quantifier and qualifier) of all the 19 drugs at 20 ng/mL in CSS.

Table 5. LLOQ and linearity summary for 19 drugs

Analyte	Precursor Ion (*m/z*)	Quantifier Ion (*m/z*)	Qualifier Ion (*m/z*)	Ion Ratio (%)	Ion Ration Window (±%)	LLOQ (ng/mL)	Linear Range (ng/mL)	R^2
Amitriptyline	278.10	202.10	233.10	105	21	4	4–400	0.9941
Bromazepam	316.11	182.10	209.10	90	18	10	10–400	0.9955
Clobazam	301.10	259.10	224.10	37	7	10	10–400	0.9967
Clomipramine	315.10	86.00	58.00	35	7	4	4–400	0.9933
Clonazepam	316.00	270.10	214.00	35	7	10	10–400	0.9960
Clozapine	327.10	270.10	192.00	70	14	4	10–400	0.9974
Diazepam	285.10	193.10	154.00	67	13	4	4–400	0.9951
Dothiepin	296.10	202.10	221.10	84	17	10	10–400	0.9937
Doxepin	280.10	165.10	107.00	180	36	4	4–400	0.9955
Flunitrazepam	314.10	268.10	239.10	39	8	4	4–400	0.9973
Imipramine	281.20	86.00	58.00	35	7	4	4–400	0.9972
Lamotrigine	256.00	211.00	109.00	50	10	10	10–400	0.9881
Levetiracetam	171.10	126.10	98.10	4.6	2	10	10–400	0.9945
Nitrazepam	282.10	236.10	207.10	35	7	4	4–400	0.9980
Nortriptyline	264.20	233.20	91.10	73	15	4	4–400	0.9948
Oxazepam	287.10	269.10	104.10	13	4	10	10–400	0.9943
Perhexilline	278.20	95.10	67.00	66	13	20	20–400	0.9755
Temazepam	301.11	255.10	283.10	25	5	4	4–400	0.9948
Trimipramine	295.20	100.10	58.10	44	9	4	4–400	0.9968

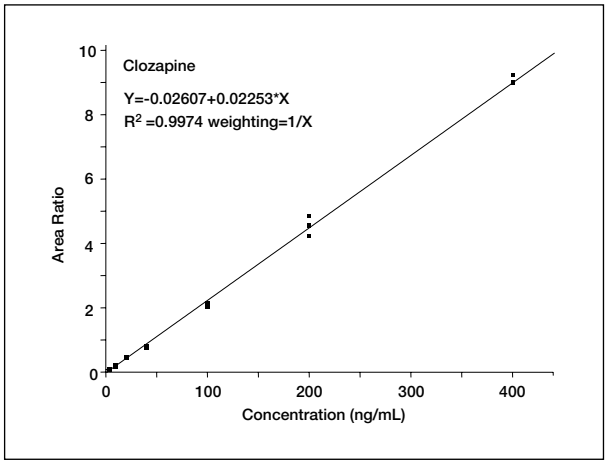

Figure 3. Calibration curve of clozapine in CSS

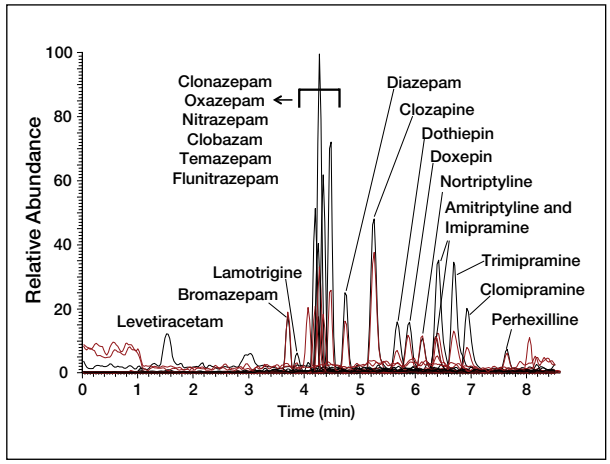

Figure 4. SRM chromatograms of all 19 drugs at 20 ng/mL in CSS after 200-fold dilution

Accuracy and Precision

Accuracy and precision were first assessed with CSS spiked at concentrations of 40 and 200 ng/mL (Table 6). Overall accuracy ranged between 82.4% and 111.3%. Inter- and intra-batch precision (coefficient of variation) values at low (40 ng/mL) and high (200 ng/mL) concentrations varied between 1.4% and 13.5%. Accuracy and intra-batch precision were also assessed in the 9 individual human-donor plasma samples spiked with 40 ng/mL drugs. The results were satisfactory (Table 7).

Table 6. Accuracy and precision summary for analysis of 19 drugs in CSS

Analyte	40 ng/mL					200 ng/mL				
	Precision				Accuracy	Precision				Accuracy
	Intra1 (%) n=5	Intra2 (%) n=5	Intra3 (%) n=5	Inter (%) n=15	Inter (%) n=15	Intra1 (%) n=5	Intra2 (%) n=5	Intra3 (%) n=5	Inter (%) n=15	Inter (%) n=15
Amitriptyline	8.5	10.4	11.5	9.7	87.8	4.6	3.8	9.7	6.3	100.7
Bromazepam	10.1	2.9	3.8	6.9	89.9	3.1	4.0	2.1	3.3	104.2
Clobazam	2.5	3.4	8.6	5.1	90.6	5.5	4.0	4.3	4.6	101.7
Clomipramine	8.3	8.1	6.4	8.0	106.3	3.2	3.1	3.1	4.8	109.4
Clonazepam	3.6	6.4	6.1	5.2	101.4	5.6	2.1	3.3	4.4	107.4
Clozapine	5.7	3.4	5.1	5.4	96.5	4.4	4.3	2.4	3.6	111.3
Diazepam	4.9	6.9	5.9	5.9	88.8	2.7	4.7	3.6	3.6	101.7
Dothiepin	3.7	8.9	5.4	6.1	99.5	4.2	2.5	4.0	4.9	108.2
Doxepin	5.8	10.8	11.9	10.0	96.4	4.5	4.5	2.9	4.5	108.8
Flunitrazepam	1.4	7.0	4.2	5.1	82.4	4.7	4.2	4.3	4.5	100.8
Imipramine	3.1	2.9	2.0	2.8	87.0	1.6	3.1	3.2	2.9	102.2
Lamotrigine	7.0	5.2	8.9	7.2	96.9	3.9	2.5	3.4	3.8	105.8
Levetiracetam	10.9	3.9	9.5	8.3	99.1	5.4	3.0	8.9	5.9	107.8
Nitrazepam	3.8	4.1	6.0	5.2	85.1	5.7	3.8	5.4	4.7	97.3
Nortriptyline	6.9	4.9	4.6	5.2	97.7	2.3	3.9	4.3	3.9	110.5
Oxazepam	8.3	5.5	9.2	7.6	96.5	5.0	7.1	1.7	5.2	106.3
Perhexilline	8.0	12.7	12.7	13.5	86.5	2.2	1.9	6.9	4.4	107.7
Temazepam	7.7	5.5	3.7	6.1	95.3	2.7	2.5	4.8	3.4	104.7
Trimipramine	3.6	3.0	6.1	4.1	89.0	2.9	3.7	3.9	3.7	103.4

Table 7. Accuracy and precision summary for analysis of 19 drugs in 9 individual human-donor plasma samples

Analyte (40 ng/mL)	Mean Measured (ng/mL), n=9	Accuracy (%) n=9	Precision (%) n=9
Amitriptyline	47.2	118.1	7.2
Bromazepam	33.7	84.3	18.0
Clobazam	42.8	107.0	15.0
Clomipramine	41.7	104.2	12.9
Clonazepam	41.4	103.4	13.4
Clozapine	38.6	96.4	9.0
Diazepam	37.2	93.1	8.8
Dothiepin	38.3	95.8	8.1
Doxepin	41.2	102.9	18.5
Flunitrazepam	34.8	87.0	7.7
Imipramine	37.4	93.4	6.5
Levetiracetam	40.0	100.1	7.7
Lamotrigine	37.2	93.0	18.2
Nitrazepam	38.9	97.3	7.0
Nortriptyline	36.2	90.5	6.2
Oxazepam	35.3	88.2	7.2
Perhexilline	42.8	106.9	9.6
Temazepam	36.1	90.3	9.1
Trimipramine	35.9	89.8	7.9

Carryover

The lowest calibrator was analyzed after the highest calibrator, and we did not observe any carryover causing elevated measurements of the drugs in the lowest calibrator.

Conclusion

We have developed a simple, fast, and sensitive LC-MS/MS clinical research method for simultaneously quantitation of 19 drugs in human plasma. The method had LLOQ values of 4–20 ng/mL for all 19 drugs and was linear to 400 ng/mL. Ion suppression was not observed in matrix samples. Accuracy and precision of the method were successfully accessed in both CSS and human plasma samples.

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