LC-MS/MS Combined with Single-Step Extraction Method for the Determination of Nicotine and Cotinine Metabolite in Rat Plasma

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1 ABSTRACT

Nicotine is a naturally occurring chemical compound that is widely used in consumer and therapeutic products. Cotinine is a major metabolite of nicotine. A liquid chromatography tandem mass spectrometry (LC-MS/MS) method for nicotine and cotinine in rat plasma has been developed. The method is based on protein precipitation extraction in a 96-well plate format and requires 25 µL of rat plasma. The analysis was carried out on a Waters UPLC®-BEH Phenyl, 1.7 µm (30 x 2.1 mm) column with a cycle time of 2 minutes. The calibration ranges are from 1 to 500 ng/mL for nicotine and cotinine. The method and slightly modified methods for various instrument platforms were qualified or validated for supporting inhalation toxicological studies from which thousands of rat plasma samples needed to be analyzed.

2 INTRODUCTION

Nicotine, a natural alkaloid, is a major constituent of Nicotiana tubacum (tobaco) plant as a tertiary amine composed of a pyridine and pyrrolidine rings. The primary in-vivo metabolite of nicotine is cotinine (Figure 1). Its harmful effects are well known, but nicotine also has positive effects. A number of quantitative LC-MS/MS methods have recently published involving the determination of nicotine and cotinine in human plasma or urine. However, most of the reported procedures use solid phase extraction or liquid-liquid extraction with single-tube procedures.1-2 Complicated procedures lead to low sample throughput and contamination due to the volatile nature of nicotine and cotinine. Reliable bioanalytical methods with high sample throughput are needed in support of toxicological studies of the commercial products containing nicotine. We describe LC-MS/MS coupled with single-step extraction method for the determination of nicotine and cotinine in rat plasma in support of ongoing toxicological inhalation studies.

3 EXPERIMENTAL

LC-MS/MS system consisted of a Waters Acquity UPLC® System coupled with API 4000 triple quadrupole mass spectrometer.

LC Method

LC Column:	Waters UPLC® BEH Phenyl, 2.1x30 mm 1.7-um
Column Temperature:	60°C
Auto-sampler Temperature:	4°C
Mobile Phase A:	2 mM NH_4HCO_3
Mobile Phase B:	2 mM NH_4HCO_3 in 95% CH_3CN
Strong Wash:	IPA :CH_3CN: MeOH (50:25:25 v/v/v)
Weak Wash:	40% MeOH
Seal Wash:	10% CH_3CN

LC Gradient Program

Compound Name	A%	B%	Flow rate (mL/min)
Initial	90	10	0.55
1.20	60	40	0.55
1.21	5	95	0.55
1.22	5	95	1.20
1.80	5	95	1.20
1.81	90	10	1.20
2.00	90	10	1.20

Mass spectrometric detection parameters (API-4000)

Compound Name	Precursor Ion m/z	Product Ion m/z	Ionization Mode
Nicotine	163.3	130	Positive
Cotinine	177.3	80	Positive
Nicotine-d4	167.3	134	Positive
Cotinine-d3	180.3	80	Positive

3 EXPERIMENTAL, continued

Sample Preparation

Rat plasma samples were prepared using a protein precipitation procedure in a 96-well format. Plasma samples (25 µL) were mixed with 200 µL of a working internal standard solution (12.5 ng/mL each in 1% $(NH_4)_2CO_3$ in MeOH). After vortex mixing and centrifugation, the organic layers (150 µL) were transferred into a clean plate. The extracted samples were diluted with water (150 µL), then injected for LC-MS/MS analysis after centrifugation.

Figure 1. Nicotiana tabacum plant and major pathway of nicotine metabolism.

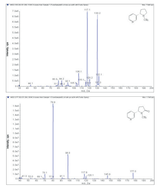

Figure 2. Product ion mass spectra (MS/MS) of the protonated nicotine and cotinine.

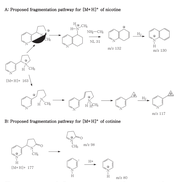

Figure 3. Proposed fragmentation pathways for protonated nicotine and cotinine under ESI collision induced dissociation (CID) conditions.

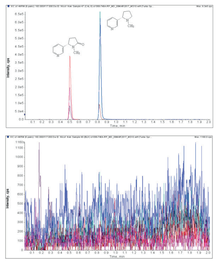

Figure 4. Representative LC-MS/MS chromatograms of extracts of ULOQ (500 ng/mL each) and rat plasma blank matrix..

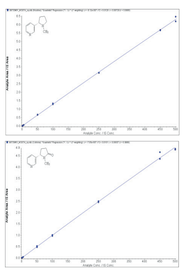

Figure 6. Calibration standard curves from 1 to 500 ng/mL for nicotine and cotinine.

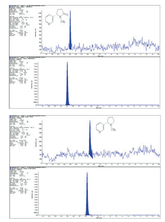

Figure 5. LC-MS/MS chromatograms of extracts of LLOQ, (1 ng/mL each analyte).

Table 1A. Precision and Accuracy of Calibration Standards of Nicotine in Rat Plasma

Calibrators	CAL 1	CAL 2	CAL 3	CAL 4	CAL 5	CAL 6	CAL 7	CAL 8
Nominal Conc. (ng/mL)	1.00	2.00	5.0	50.0	100	250	450	500
Data Point #1	0.94	1.85	4.96	51.1	99.8	246	449	491
Data Point #2	1.10	2.04	4.77	52.1	104	247	448	513
Mean	1.02	1.94	4.86	51.6	102	247	448	502
Standard Dev.	0.11	0.14	0.13	0.67	2.8	0.59	1.1	16
%CV	10.7	6.98	2.67	1.3	2.7	0.24	0.24	3.2
Accuracy	101.9	97.2	97.3	103	102	98.6	99.6	100
Number of Values	2 of 2	2 of 2	2 of 2	2 of 2	2 of 2	2 of 2	2 of 2	2 of 2

Table 1B. Precision and Accuracy of Quality Control Samples of Nicotine in Rat Plasma

Quality Controls	QC 1	QC 2	QC 3	QC 4
Nominal Conc. (ng/mL)	1.00	5.00	100	400
Intra-run Mean (ng/mL)	0.982	4.96	98.5	454
Standard Dev.	0.10	0.36	1.6	10.6
%RSD	10.5	7.2	1.7	2.3
%RE	98.2	99.2	98.5	101
Number of Values	6	6	6	6

Table 2A. Precision and Accuracy of Calibration Standards of Cotinine in Rat Plasma

Calibrators	CAL 1	CAL 2	CAL 3	CAL 4	CAL 5	CAL 6	CAL 7	CAL 8
Nominal Conc. (ng/mL)	1.00	2.00	5.0	50.0	100	250	450	500
Data Point #1	0.90	2.10	4.80	47.6	96.9	252	445	488
Data Point #2	1.05	2.14	4.87	52.6	101	247	476	492
Mean	0.98	2.12	4.84	50.1	99.1	249	461	490
Standard Dev.	0.11	0.03	0.05	3.50	3.2	3.14	21.9	2.9
%CV	10.9	1.36	0.93	6.99	3.2	1.26	4.76	0.6
Accuracy	97.7	106	96.7	100.2	99.1	99.8	102.4	98.1
Number of Values	2 of2	2 of2	2 of 2	2 of 2	2 of 2	2 of2	2 of 2	2 of 2

Table 2B. Precision and Accuracy of Quality Control Samples of Cotinine in Rat Plasma

Quality Controls	QC 1	QC 2	QC 3	QC 4
Nominal Conc. (ng/mL)	1.00	5.00	100	400
Intra-run Mean (ng/mL)	1.17	5.07	97.5	456
Standard Dev.	0.16	0.19	2.4	10.5
%RSD	14	3.8	2.5	2.3
%RE	117	101	97.5	101
Number of Values	6	6	6	6

4 RESULTS AND DISCUSSION

ESI-MS/MS Detection of Nicotine and Cotinine

Nicotine and cotinine are both bicyclic compounds with pKa values 3.8, 8.6 for nicotine and 4.8, -0.5 for cotinine (Figure 1). Nicotine gives its pseudomolecular ion [M+H]+, at m/z 163.2 and it fragments in Q2 to give two major product ions m/z 130.2 and m/z 117.1 (Figure 2 A). Cotinine gives a pseudomolecular ion [M+H]+ at m/z 177.3 and it produces two major fragments at m/z 80 and m/z 98 (Figure 2B). Based on their structures and functional group properties, the proposed fragmentation pathways are presented in Figure 3.

LC-MS/MS Analysis of Nicotine and Cotinine

Rat plasma samples (25 µL aliquots) were extracted with 1% ammonium carbonate in methanol (pH ~10) to precipitate plasma proteins and to release the protein bound nicotine and cotinine. After adding water, plasma extracts were injected on to a BEH phenyl, 1.7 µm (30 x 2.1 mm) column with a run time of 2 minutes (Figure 4A). The simple one-step plasma sample extraction avoids loss and cross contamination of volatile compounds such as nicotine and cotinine (Figure 4B). Additionally, using basic mobile phase conditions (2 mM ammonium carbonate), deprotonated nicotine and cotinine eluted with higher organic content which leads to higher sensitivity of electrospray mass spectrometric detection (Figure 5). Typical calibration standard curves for nicotine and cotinine both from 1 to 500 ng/mL in rat plasma are shown in Figure 6. Precision and accuracy data are listed in Table 1 and Table 2 for both analytes. Method precision and accuracy were acceptable based on the CRL SOP established criteria.

5 CONCLUSION

We have developed a simple, reliable, and high throughput LC-MS/MS method for simultaneous quantification of nicotine and metabolite cotinine from 1 to 500 ng/mL in rat plasma. Modified LC-MS/MS methods have been qualified or validated and used for analyses of thousands of real samples from inhalation toxicological studies. The general method methodologies can also be extended to nicotine and its related metabolites in other biological matrices.

REFERENCES

1. M. Piller, G. Gilch, G. Scherer and M. Scherer, Journal of Chromatography B 951-951 (2014) 7-15.
2. Sponsor internal method validation report: Nicotine Analysis in Human Plasma.

ACKNOWLEDGEMENTS

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