Determination of Cotinine by LC—MS-MS with Automated Solid-Phase Extraction

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Cotinine is the primary metabolite of nicotine and the preferred biomarker for assessing cigarette smoke exposure. Several liquid chromatography—tandem mass spectrometry (LC—MS-MS) methods have been described for measuring cotinine in biological fluids. Sample preparation typically involves manual solvent evaporation and reconstitution steps. This study describes a novel LC—MS-MS method for the quantification of cotinine by using electrospray ionization with multiple reaction monitoring and cotinine-d₃ as internal standard, coupled with an automated solid-phase extraction (SPE) procedure. The assay was linear over the analytical range of 0.5–1,000 ng/mL. The limits of detection and quantification were 0.13 and 0.20 ng/mL, respectively. Intra-assay and inter-assay imprecision of cotinine in all samples was <5 and <10% (coefficient of variation), respectively. The analytical recovery of cotinine spiked into plasma was >95–100%. Matrix effects in serum and plasma were <10%.

A rapid, sensitive and specific LC—MS-MS method was developed and validated for the determination of cotinine in human plasma, using a straightforward automated SPE protocol. The application of this method to an epidemiological study has demonstrated its utility for batch analyses of a large sample set (>500 samples).

Introduction

The adverse effects of cigarette smoking on health are widely appreciated, particularly in pulmonary disease, cardiovascular disease and cancer (1–3). In studies linking tobacco smoke exposure to health outcomes, the biochemical analysis of nicotine and its metabolites is often required to provide a reliable measure of exposure to cigarette smoke (4).

Following inhalation of cigarette smoke, nicotine is rapidly absorbed into the bloodstream and metabolized in the liver (5). Cotinine is the major primary metabolite, has a much longer half-life and is present in the blood at much higher levels than nicotine (5). Consequently, cotinine is the biochemical marker of choice for measuring exposure to cigarette smoke and has been widely used in quantitative studies of smoking status/behavior (6).

To date, several methods have been developed to measure cotinine in a variety of biological matrices, including blood (serum or plasma), urine, saliva, hair and meconium (6—9). Liquid chromatography—tandem mass spectrometry (LC—MS-MS) methods have predominated, owing to the increased sensitivity afforded by these instruments, which permits lower limits of quantification ≤0.05 ng/mL in plasma (10, 11). Importantly, this allows the assessment of passive smoke exposure in non-smoking individuals and the potential health effects thereof. However, current protocols typically employ lengthy sample preparation steps, often requiring solvent evaporation and reconstitution (10—14). The method presented herein preserves the sensitivity afforded by LC—MS-MS and describes a rapid, novel and straightforward automated solid-phase extraction (SPE) protocol.

The method is particularly suited to the analyses of large sample numbers, typically associated with studies linking smoking behavior to health, and potentially for routine use in clinical laboratories. As proof of principle, the method was applied for the validation of self-reported smoking status/behavior in >500 samples from participants in an epidemiological study.

Experimental

Materials

Cotinine (1 mg/mL dissolved in methanol) and tri-deuterated cotinine (cotinine-d₃; 1 mg/mL dissolved in methanol) were purchased from LGC Standards (Middlesex, UK). Ammonium acetate, formic acid and bovine serum were purchased from Sigma-Aldrich (Poole, UK). All other solvents were high-purity analytical grade and purchased from Rathburn Chemicals (Walkerburn, UK). Microliter C8 ITSP cartridges for SPE were purchased from Preseach (Basingstoke, UK).

Preparation of calibration standards and quality controls

A stock solution of 1,000 mg/L of cotinine was prepared in methanol and stored at 4°C. Working solutions (0.001–10 mg/L) were prepared by serial dilution in deionized water. Blank bovine serum samples were spiked with 25, 50 or 100 μL aliquots of working solutions to yield a seven-point calibration curve (0.5, 1, 10, 100, 250, 500 and 1,000 ng/mL) for cotinine. A stock solution of 1,000 mg/L of cotinine-d₃ internal standard (IS) was prepared in methanol and stored at 4°C. A working solution of cotinine-d₃ (1,000 ng/mL) was prepared by serial dilution in deionized water. A 50 μL aliquot of the IS working solution was added to 1 mL of each standard before use. A zero standard was prepared in blank bovine serum including IS, but with no added cotinine.

Two levels of quality control (QC) materials were prepared in-house by spiking bovine serum with working solutions of cotinine. Bovine serum samples were spiked with 200 μL of 0.1 mg/L or 400 μL of 10 mg/L cotinine working solutions to yield final concentrations of 2 and 400 ng/mL (low QC and high QC, respectively). QC material was stored at ~20°C in 1 mL aliquots. Fifty microliters of IS working solution was added to 1 mL of each QC sample prior to use.
Sample preparation
Fifty microliters of IS working solution were added to 1 mL of each human plasma, QC or calibrator sample in a 5 mL glass screw-top diluent tube. The tubes were capped and mixed end-on-end for 10 min. Each sample was transferred to a 2 mL screw-top vial and loaded into the sample tray of an HTS-PAL automated sampler robot (CTC Analytics AG), which was equipped with ITSP SPE cartridges. The HTS-PAL robot was pre-programmed to perform SPE in a five-step procedure, taking approximately 4 min per sample: a C8 ITSP cartridge was conditioned with 150 µL methanol, followed by 150 µL deionized water. Next, 400 µL of plasma sample was loaded onto a C8 ITSP cartridge and washed with 150 µL of 10% (v/v) methanol in deionized water. Cotinine and cotinine-d3 were eluted into 0.3 mL screw-top vials with 200 µL methanol. Sample vials were manually capped, vortex mixed and loaded into the Waters Xevo autosampler tray.

LC–MS–MS analysis
LC–MS–MS analysis was performed on a Waters Acquity ultra-performance liquid chromatography (UPLC) system interfaced with a Waters Xevo TQ MS (Waters, Manchester, UK). The entire system was controlled by Mass Lynx v4.1 software. Chromatographic separation of cotinine and cotinine-d3 was achieved with an Acquity UPLC BEH C8 column (2.1 × 50 mm; 1.7 µm particle size). The temperature of the column was maintained at 60 ºC. A portion of 2.5 µL of the extracted sample was injected onto the column and gradient elution was performed with 5 mmol/L ammonium acetate and 0.05% (v/v) formic acid in deionized water (mobile phase A) and (methanol mobile phase B) at a flow rate of 0.35 mL/min. MS detection of cotinine and cotinine-d3 was conducted by electrospray ionization (ESI) in the positive ion mode, using multiple reaction monitoring (MRM) for analyte identification. The following ESI conditions were applied: capillary voltage of 50 kV; source temperature of 150 ºC; desolvation temperature of 500 ºC; desolvation gas flow (nitrogen) of 1,000 L/h; collision gas flow (argon) of 0.15 mL/min. Tuning of the MS was conducted with the Intellistart feature of Mass Lynx v4.1, by infusing 1 mg/L solutions of cotinine and cotinine-d3 directly into the MS at 20 µL/min. The MRM transitions monitored were as follows: cotinine (m/z 177 → 80, 177 → 98 and 177 → 146), cotinine-d3 (m/z 180 → 73 and 180 → 100). The analysis time was approximately 4 min per sample.

Data analysis
The Targetlynx feature of Mass Lynx v4.1 software was used to generate calibration curves and calculate concentrations based upon the peak area ratios of cotinine and cotinine-d3. The most abundant transition for each analyte was used for quantification (cotinine: m/z 177 → 80; cotinine-d3: m/z 180 → 73) and a second transition served as a qualifier (cotinine: m/z 177 → 98). Data were fit to a linear least-squares regression curve with a weighting factor of 1/\(x\), using exclude origin.

Method validation
The method was validated in accordance with published criteria (15–16). It was difficult to find a certified source of cotinine-free human plasma or serum. Consequently, bovine serum was chosen as the matrix for method development, which was demonstrated to be negative for both nicotine and cotinine.

The selectivity of the method was assessed by analyzing bovine serum spiked with cotinine at concentrations across the analytical range (0–1,000 ng/mL) and confirmed based upon relative retention times and quantifier/qualifier transition peak area ratios. Potential interference from nicotine was evaluated by fortifying bovine serum with nicotine at concentrations spanning the analytical range. Potential carryover between injections was investigated by the analysis of bovine serum fortified with cotinine-d3 after the analysis of the highest calibration.

The sensitivity of the method was determined by the lower limit of detection (LLOD) and lower limit of quantification (LLOQ), which were calculated relative to peak area. To determine the LLOD, five replicates of the zero standard were analyzed and the mean and standard deviation (SD) were calculated. The concentration of cotinine that corresponded to the mean +3SDs was estimated to be the LLOQ. The LLOQ was statistically determined as the cotinine concentration at which the analyte peak was equivalent to 10 SDs of the zero standard.

The linearity of the method was determined by analyzing eight calibrators ranging from 0 to 1,000 ng/mL. A standard curve was constructed and the regression line was calculated by the method of least squares with 1/x weighting. The response was considered to be linear if the correlation coefficient (\(r^2\)) was ≥ 0.99. Calibrator concentrations were required to be within ±15% of the target concentration (15).

The intra-assay imprecision for cotinine was determined at seven concentrations across the analytical range of the assay: 0.5, 1, 10, 100, 250, 500 and 1,000 ng/mL. Intra-assay data were assessed by comparing data from within a run (n = 5). Data on inter-assay imprecision were determined from a total of 12 replicates for each QC assayed on 12 separate runs. The mean, SD and coefficient of variation (CV) were calculated. Imprecision was expressed as CV (%).

The analytical recovery was determined by comparing the concentration of cotinine in bovine serum samples before (base sample) and after (spiked sample) the addition of known amounts of cotinine (spike added). Two bovine serum samples containing 1 and 500 ng/mL of cotinine were spiked with 0.5 and 50 ng/mL of cotinine, respectively. Recovery was calculated by using the following equation: Recovery (%) = (spiked sample – base sample/spike added) × 100.

The matrix effect on the ionization of cotinine and cotinine-d3 was quantitatively assessed in both bovine serum and human plasma by using two sets of samples (17). In the first set, bovine serum and human plasma samples were spiked with cotinine and cotinine-d3 following SPE. In the second set, methanol was spiked with cotinine and cotinine-d3 at an equivalent concentration to Set 1. The matrix effect was calculated by dividing the instrument response (calculated from peak area ratios of cotinine to cotinine-d3) of cotinine in Set 1 by the instrument response in Set 2. The value was subtracted from 1 and converted to a percentage to represent the amount of signal enhancement (positive) or suppression (negative) by the matrix. Matrix effects were also assessed qualitatively by the post-column infusion method (18).

Stability was assessed by using bovine serum samples fortified with cotinine and cotinine-d3 across the analytical range (0.5–1,000 ng/mL). The temperature stability was tested by
repeat analyses of samples following storage in the autosampler (10°C) for 48 and 72 h. Samples were considered stable if the measured values remained within 10% of the initial concentration (16). Specimen suitability was evaluated by analysing blood samples from smokers and non-smokers collected in lithium heparin, potassium EDTA and serum separator tubes.

Results

Selectivity

The retention time for cotinine and cotinine-$d_3$ was 0.78 min within a total chromatographic run time of 4 min (Figure 1). Peak homogeneity for each analyte was confirmed by quantifier/qualifier transition peak area ratios (acceptance limits were ±20% of the ion ratio of the 0.01 mg/L standard). A peak corresponding to nicotine was observed at 0.46 min, which did not interfere with the quantification of cotinine or cotinine-$d_3$. The MS method was developed following the direct infusion of cotinine and cotinine-$d_3$ in methanol; the optimized parameters are shown in Table I. Blank samples injected after the highest calibrator did not show any carryover.

Linearity

Calibration curves for cotinine were constructed by plotting the nominal cotinine concentration against the measured cotinine concentration. The calibration curve for cotinine was linear across the analytical range from 0.5 to 1,000 ng/mL with $r^2 >0.99$, and was reproducible between batches (Figure 2).

Sensitivity

The LLOD can be defined as the lowest amount of analyte in a sample that can be distinguished from background noise, with a signal-to-noise (S/N) ratio of 3:1 (15). The LLOQ can be defined as the lowest analyte concentration that can be quantified with an S/N ratio of 10:1 (19). The LLOD was established at 0.13 ng/mL and the LLOQ at 0.20 ng/mL. The LLOQ is further supported by the data in Figure 3, in which the CV of the lowest calibrator (0.5 ng/mL) was acceptable for quantification on the calibration curve (<5% CV).

Imprecision

Figure 2 and Table II show imprecision data for bovine serum samples fortified with cotinine and cotinine-$d_3$. Intra-assay and inter-assay imprecision were both acceptable for cotinine at <5 and <10%, respectively. Inter-assay imprecision was further supported by the data outlined in Figure 3, which shows five replicates of five calibrator concentrations, each with CV <5%.

Recovery

The recovery of cotinine was found to be greater than 95% at two concentrations (low: 1 ng/mL; high: 500 ng/mL) relative to the calibration range. Results are shown in Table III.

Matrix effect

The influence of bovine serum and human plasma upon the ionization of cotinine was performed as described previously. No ion suppression was detected for cotinine in either matrix; however, a small ion enhancement effect was observed in both bovine serum (8.9%) and human plasma (7.3%; Figure 4). Because these effects were less than 10%, they were considered acceptable for this study (20).

Stability

The stability of cotinine in bovine serum was assessed by repeat analyses of three samples following the storage protocol intended for method application. Therefore, cotinine stability was tested after storage in the autosampler (10°C) for 48 and 72 h. Cotinine concentrations at low (0.5 ng/mL), medium (100 ng/mL) and high (1,000 ng/mL) relative to the analytical range were within ±5% of expected values, indicating sample stability under these conditions (Table IV). To demonstrate the applicability of the method, plasma and serum samples from known smokers and non-smokers were collected and analyzed for cotinine. The results were consistent with those expected of
a non-smoker (<10 ng/mL) and an active smoker (>10 ng/mL) (4, 21). Furthermore, results were within ±5% of one another, indicating that either lithium heparin, potassium EDTA or serum separator blood collection tubes were suitable for cotinine analysis by this method.

**Discussion**

The aim of this study was to develop and validate a rapid, sensitive and specific LC–MS-MS method suitable for the quantification of cotinine in human plasma. In particular, the method was required to be practical for the analysis of many samples from an epidemiological study. Therefore, a key objective was to develop an automated SPE procedure that would facilitate high-throughput analyses.

Several LC–MS-MS methods have been described in the literature for the measurement of cotinine in serum/plasma (7, 12–14, 22). As expected of such a sensitive technique, the LLOD and LLOQ for cotinine are typically reported in the low ng/mL range, with the most sensitive assays reporting LLOQ < 0.05 ng/mL in plasma (10, 1). However, almost all published methods have used sample preparation and extraction protocols that require solvent evaporation and reconstitution (12–14, 22).

In this study, a rapid LC–MS-MS method has been developed for the quantification of cotinine in human plasma. LC–MS-MS analysis of cotinine and cotinine-d₃ are both complete within 4 min per sample. Indeed, both analytes elute within the first minute (0.78 min), so there is potential to reduce the run time even further. The assay is linear over the analytical range (0.5 to 1,000 ng/mL, r² > 0.99), which encompasses the spectrum of cotinine concentrations encountered in both active smokers and non-smokers (4, 5). The LLOD (0.13 ng/mL) and LLOQ (0.20 ng/mL) are comparable with the most sensitive plasma cotinine assays reported previously and are significantly below

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**Table I**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Parent (m/z)</th>
<th>Daughter (m/z)</th>
<th>Dwell (s)</th>
<th>Cone (V)</th>
<th>Collision (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotinine</td>
<td>177</td>
<td>80</td>
<td>0.157</td>
<td>32</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>177</td>
<td>98</td>
<td>0.157</td>
<td>32</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>177</td>
<td>146</td>
<td>0.157</td>
<td>32</td>
<td>16</td>
</tr>
<tr>
<td>Cotinine-d₃</td>
<td>180</td>
<td>73</td>
<td>0.157</td>
<td>34</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>100</td>
<td>0.157</td>
<td>34</td>
<td>20</td>
</tr>
</tbody>
</table>

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**Table II**

<table>
<thead>
<tr>
<th>Sample (ng/mL)</th>
<th>Number of replicates</th>
<th>Mean cotinine (ng/mL)</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low QC (2)</td>
<td>12</td>
<td>1.78</td>
<td>0.02</td>
<td>5.23</td>
</tr>
<tr>
<td>High QC (400)</td>
<td>12</td>
<td>372.28</td>
<td>0.02</td>
<td>5.23</td>
</tr>
</tbody>
</table>

**Table III**

<table>
<thead>
<tr>
<th>Cotinine (ng/mL)</th>
<th>Base sample</th>
<th>Spike added</th>
<th>Spiked sample</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set 1 (low)</td>
<td>0.9</td>
<td>0.4</td>
<td>1.3</td>
<td>100</td>
</tr>
<tr>
<td>Set 2 (high)</td>
<td>482.6</td>
<td>43.8</td>
<td>524.4</td>
<td>95.4</td>
</tr>
</tbody>
</table>

**Figure 2.** Nominal cotinine concentration (ng/mL) versus measured cotinine concentration (ng/mL). Plot of mean (n = 5) cotinine concentration measured across seven points on the calibration curve (0.5, 1, 10, 100, 250, 500 and 1,000 ng/mL); 95% confidence intervals are shown (dotted line).

**Figure 3.** Scatterplot of mean cotinine concentration versus CV (%). Plot of mean (n = 5) cotinine concentration measured across five points on the calibration curve (0.5, 1, 10, 500 and 1,000 ng/mL); 95% confidence intervals are shown (dotted line).
The lowest cut-off point (3 ng/mL) suggested for assessing smoking status (4).

The evaluation of intra-assay and inter-assay imprecision, recovery, stability and matrix effects all fulfilled the requirements for method validation (15, 16).

A straightforward automated SPE procedure was also devised to expedite the analytical process. Optimal SPE conditions were determined by assessing the extraction efficiency of several combinations of ITSP cartridge and solvents. The extraction efficiency was evaluated by a comparison of cotinine peak area ratios in each tested condition. The optimal wash and elution solvents were 10% (v/v) methanol in deionized water and methanol, respectively, in combination with C8 ITSP cartridges.

The automated SPE procedure described herein significantly streamlines sample preparation and improves upon a similar manual protocol previously described by Byrd et al. (25). This five-step protocol performs SPE in 4 min per sample, requiring only 400 µL of plasma and minimal amounts of solvent. The readily available solvents are cheap, and elution in methanol permits direct injection into the LC–MS-MS instrument. Thus, with the exception of calibrator preparation, manual pipetting is kept to a minimum. In addition, the process is highly reproducible and less prone to error than traditional multistage liquid–liquid extraction or SPE methods. The liquid-handling robot sample tray accommodates 48 vials, making it ideal for batch analyses.

The automated SPE robot is a stand-alone instrument, meaning that samples must be manually transferred to the MS once SPE is complete. Many instruments are now manufactured with built-in SPE modalities to circumvent this issue. However, the LC–MS-MS instrument is in routine use as part of the clinical service of the laboratory; in these circumstances, the ability to perform offline SPE can be advantageous. In this study, batches of samples were subjected to SPE while the MS instrument performed the clinical workload. Thereafter, study samples could be run in batches without disruption to the clinical service. To this end, the short-term stability studies demonstrated that samples could be stored between runs. However, the large sample set used in this study had been stored at –80°C for >12 months; thus, the long-term stability of cotinine in these samples is still under evaluation. Reassuringly, it has been reported in the literature that long-term storage at –80°C is not detrimental for plasma cotinine measurement (23).

To date, there is no external quality assessment (EQA) scheme in the UK for serum/plasma cotinine. Consequently, accuracy or bias could not be evaluated based on a comparison with another laboratory and/or method. As a result, the evaluation of accuracy was limited to recovery experiments in bovine serum. The lack of a certified cotinine-free human serum/plasma was also prohibitive in this regard. However, bovine serum proved to be a good substitute for human plasma during method development and has been similarly employed in other studies (12).

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Method application

The method described herein was successfully applied to the validation of self-reported smoking status/behavior in > 500 plasma samples from participants in an epidemiological study (24). The plasma cotinine concentration in this cohort ranged from 0 to 784 ng/mL, which was consistent with the spectrum of cigarette smoke exposure and within the linear range of the assay. The measurement of plasma cotinine concentration demonstrated that self-reporting underestimated smoking in this group by 3.5% (unpublished data).

Conclusions

This study developed and validated a rapid, sensitive and specific LC–MS–MS method for the quantification of cotinine in human plasma. The use of UPLC in this study significantly improves the sensitivity of the assay. The LLOQ is 10-fold beneath the recommended 3 ng/mL cut-off point to distinguish smokers from non-smokers. Furthermore, the linear range of this assay spans the full scope of plasma cotinine concentrations encountered with varying cigarette smoke exposure. The application of this method has facilitated the high-throughput analysis of a large sample set from participants in an epidemiological study, permitting the validation of self-reported smoking status.

Acknowledgments

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