Simultaneous determination of cotinine and trans-3-hydroxycotinine in urine by automated solid-phase extraction using gas chromatography–mass spectrometry

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ABSTRACT: A gas chromatography–mass spectrometry method was developed and validated for the simultaneous automated solid-phase extraction and quantification of cotinine and trans-3-hydroxycotinine in human urine. Good linearity was observed over the concentration ranges studied ($R^2 > 0.99$). The limit of quantification was 10 ng/mL for both analytes. The limits of detection were 0.06 ng/mL for cotinine (COT) and 0.02 ng/mL for trans-3-hydroxycotinine (OH-COT). Accuracy for COT ranged from 0.98 to 5.28% and the precision ranged from 1.24 to 8.78%. Accuracy for OH-COT ranged from −2.66 to 3.72% and the precision ranged from 3.15 to 7.07%. Mean recoveries for cotinine and trans-3-hydroxycotinine ranged from 77.7 to 89.1%, and from 75.4 to 90.2%, respectively. This analytical method for the simultaneous measurement of cotinine and trans-3-hydroxycotinine in urine will be used to monitor tobacco smoking in pregnant women and will permit the usefulness of trans-3-hydroxycotinine as a specific biomarker of tobacco exposure to be determined. © 2014 The Authors. Biomedical Chromatography published by John Wiley & Sons Ltd.

Keywords: cotinine; trans-3-hydroxycotinine; urine; GCMS; SPE

Introduction

Smoking is an unquestionable risk factor for disease and consequent death because there are many toxic chemical substances in cigarette smoke (Smith and Hansch, 2000; Hammond and O’Connor, 2008). Nicotine is the main active ingredient of tobacco and is the principal contributor that leads to smoking dependence. Because the blood levels of nicotine decrease rapidly, cotinine, a major degradation product of nicotine metabolism, is considered a specific biomarker for evaluating cigarette smoke exposure (Benowitz and Jacob, 2009). Its half-life is a much longer (16–20 h) than that of nicotine (2 h), is identical in biological fluids and presents higher concentrations than nicotine (Benowitz, 1996; Benowitz and Jacob, 2009). Cotinine is hydroxylated to give trans-3-hydroxycotinine which is eliminated predominantly as unchanged drug (Bao et al., 2005; Benowitz et al., 2009). The measurement of the ratio cotinine/trans-3-hydroxycotinine in smokers makes it possible to explore on a large scale the metabolism of nicotine. Such calculation is likely to facilitate links between the catabolism of nicotine and the criteria of assessment of dependency and consumption (St Helen et al., 2013; Murphy et al., 2013). However, urinary cotinine and trans-3-hydroxycotinine are the preferred markers because urine collection is noninvasive. Several methods are used to quantify nicotine and metabolites in human urine, including ultraperformance liquid chromatography, liquid chromatography–mass spectrometry, gas chromatography–mass spectrometry (GCMS), gas chromatography with nitrogen phosphorus detection and immunoassays (Shulgin et al., 1992; Ji et al., 1999; Meger et al., 2002; Moyer et al., 2002; Xu et al., 2004; Chao et al., 2005; Heavner et al., 2005; Kim et al., 2005; Song et al., 2005; Hoofnagle et al., 2006; Kataoka et al., 2009; Shakley and Huestis, 2009a and 2009b; Baumann et al., 2010; Hu et al., 2010; Malaffati et al., 2010; Marclay and Saugy, 2010; Miller et al., 2010; Dobrinas et al., 2011; Jacob et al., 2011; Rangiah et al., 2011; Kuhn et al., 2012; Scheidweiler et al., 2012). Older methods include quantification of cotinine and trans-3-hydroxycotinine in urine by GCMS (Shulgin et al., 1992; Ji et al., 1999; Kim et al., 2005). This paper reports an automated solid-phase extraction (SPE) procedure to determine simultaneous quantification of cotinine and trans-3-hydroxycotinine by GCMS across a wide range of concentrations in human urine.

Experimental

Materials

Cotinine (COT), trans-3-hydroxycotinine (OH-COT), cotinine-d3 and trans-3-hydroxycotinine-d3 were obtained from LGC Standards (Molsheim, France). BSTFA (bistrimethylsilyl) trifluoroacetamide; COT, cotinine; OH-COT, trans-3-hydroxycotinine; SPE, solid-phase extraction; TMS, trimethylchlorosilane

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**Abbreviations used:** BSTFA, bistrimethylsilyl) trifluoroacetamide; COT, cotinine; OH-COT, trans-3-hydroxycotinine; SPE, solid-phase extraction; TMS, trimethylchlorosilane

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France). Caffeine, ibuprofen, methadone, morphine, pseudoephedrine, codeine, cocaine, clomipramine, propranolol and \(\Delta 9\)-tetrahydrocannabinol analytical reference standards presented in methanol were obtained from Cerilliant (Sigma-Aldrich, Saint-Quentin-Fallavier, France). Potassium dihydrogen phosphate and dipotassium hydrogen phosphate were obtained from Sigma-Aldrich (St Louis, MO, USA). Methanol, hexane, isopropanol were of analytical grade and were obtained from Merck (Darmstadt, Germany). Sterile water was obtained from Fresenius (Sevres, France). Ammonia solution 25% was purchased from VWR (Fontenay-sous-Bois, France). Hydrochloric acid 0.2 M was obtained from VWR (Fontenay-sous-Bois, France). Clean Screen SPE columns 200 mg were purchased from UCT (Bristol, PA, USA). The derivatizing agent utilized was bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMS) (Sigma-Aldrich, Saint-Quentin-Fallavier, France).

Blank, standard and quality controls

Urine samples from nonsmoker volunteers were collected, extracted and analyzed. Urine samples with nondetectable COT and OH-COT were pooled and used in the preparation of calibration and quality control samples (QCs). The commercial solutions containing 1 mg/mL of COT and OH-COT in methanol were used as stock solutions. Three working solutions (1, 10 and 100 \(\mu\)g/mL) were prepared from the stock solutions. A set of eight calibrators made up of 25, 50, 100, 500, 1000, 2000, 4000 and 6000 ng/mL COT and OH-COT in urine was prepared daily from working solutions. Three QCs (30, 2500 and 5000 ng/mL) of COT and OH-COT in urine were also prepared from a separate stock solution (1 mg/mL). The internal standards, cotinine-d3 and trans-3-hydroxycotinine-d3, were mixed in methanol to prepare the internal standards working solution (10 \(\mu\)g/mL). All solutions, blank and QCs were stored at \(-20^\circ\text{C}\) prior to analysis.

Sample preparation

An aliquot of 2 mL of urine sample, QC sample or calibration standard was mixed with 2 mL of 0.1 M phosphate buffer (pH 6.0). Twenty microliters of internal standards working solution were added to each sample prior to extraction. The SPE columns were preconditioned in the following order, 3 mL of methanol, 3 mL of sterile water, and 1 mL of phosphate buffer (pH 6.0). Each sample was loaded onto SPE column at 1 mL/min and washed with 3 mL of sterile water, 2 mL of 0.2 M hydrochloric acid, 2 mL of hexane and 3 mL of methanol. Analytes were eluted with 1 mL dichloromethane–ethyl acetate–hexane–methanol (78:20:2). This SPE protocol was entirely automated using the Gilson GX-271 ASPEC system. Extracts were evaporated to dryness under a stream of nitrogen at 35°C using a Thermec Di-Block® DB-3D Evaporator. Residues were reconstituted in 20 \(\mu\)L of ethyl acetate and 20 \(\mu\)L of BSTFA (with 1% TMS) and derivatized at 70°C for 30 min. The derivatized extract (1 \(\mu\)L) was injected onto the GCMS with selected ion monitoring mode.

Gas chromatography–mass spectrometry

The Thermo Focus DSQ II gas chromatograph/mass spectrometric system was used for GC separation and detection. Helium was used as a carrier gas at a constant flow of 1.20 mL/min. The system was equipped with an Uptibond® UBS premium column (30 m × 0.25 mm × 0.25 \(\mu\)m). The instrument was programmed from 70 to 190°C at 30°C/min, to 230°C at 5°C/min, and to 290°C at 25°C/min, for a total analysis time of 15 min. The transfer line temperature was maintained at 290°C. One microliter of derivatized extract was injected. The injection port temperature was held at 250°C and operated in the pulsed splitless mode. The instrument utilized electron impact ionization and was operated in the selected ion monitoring mode. The ions for each analyte were monitored in the following elution order for the derivatized analytes: cotinine-d3, m/z (122), 179; cotinine, m/z (119), 176; trans-3-hydroxycotinine-d3, m/z (147), 252; and trans-3-hydroxytrans-cotinine, m/z (249), 144.

Data analysis

Data were collected and analyzed utilizing Thermo Electron GCMS Solution software (Xcalibur™, version 1.4.2; Thermo Electron Corporation, San Jose, CA, USA).

Selectivity

Five blank urine samples from different nonsmokers were extracted and analyzed for assessment of potential interferences that may be present owing to passive smoke exposure. Potential interferences from 10 commonly used drugs were evaluated by adding compounds at concentrations of 0.2, 0.5 and 10 \(\mu\)g/mL (caffeine, ibuprofen, methadone, morphine, pseudoephedrine, codeine, cocaine, clomipramine, propranolol and \(\Delta 9\)-tetrahydrocannabinol).

Linearity and sensitivity

The linearity of the method was determined by linear regression of calibrator concentrations vs peak area ratio of either COT or OH-COT peak area divided by the peak area of the respective internal standard. The linearity of the curves was accepted when each of the calibrators achieved concentration not exceeding 20% of the nominal actual concentration. The sensitivity of the assay was evaluated by determining the limit of detection (LOD) and the limit of quantification (LOQ). The LOD is the lowest concentration of an analyte determined with signal-to-noise of at least 3:1 by peak area. The LOQ is the lowest concentration of an analyte in a calibration curve and it may use the criteria of LOQ.

Precision and accuracy

Inter- and intra-assay precision and accuracy data for COT and OH-COT were calculated by using low (50 ng/mL), medium (600 ng/mL) and high (5000 ng/mL) QC samples. Intra-assay data were assessed by comparing data from within one run (\(n = 9\)). Inter-assay data were determined by comparing data between six runs. Precision was expressed as percentage relative standard deviation (RSD), and accuracy was expressed as the deviation (%) of the mean concentration from nominal concentration.

Recovery

Recovery was determined by adding analytes to a series of six replicates in urine with low (10 ng/mL), medium (1000 ng/mL) and high concentrations (6000 ng/mL) of each analyte that were extracted and compared nonextracted standards in methanol. Recovery was expressed as a percentage of the mean peak area of the extracted replicates divided by the mean peak area of nonextracted replicates.

Stability

The stability of samples in the autosampler tray was tested by comparing blank urine samples spiked with low (50 ng/mL), medium (600 ng/mL) and high concentrations (5000 ng/mL) of each analyte and internal standards. The spiked samples were by the procedures described above followed by evaporation, reconstitution with 20 \(\mu\)L ethyl acetate and 20 \(\mu\)L BSTFA with 1% TMS, transfer to vial insert, and placement in the autosampler tray. One microliter of the solution was injected every hour from the same sample. The response ratios of each analyte and its internal standard of successive injections were compared with that of the first injection.

Results

Method validation

A typical chromatogram for the two analytes and their internal standards can be found in Fig. 1. Figure 1 shows that first cotinine-d3 is
eluting at 7.37 min, followed by cotinine at 7.39 min, trans-3-hydroxycotinine-d3 at 8.87 min, and trans-3-hydroxicotinine at 8.89 min.

**Linearity and sensitivity**

Cotinine and trans-3-hydroxycotinine were linear over the range 10–6000 ng/mL with $R^2 > 0.997$ and $R^2 > 0.999$, respectively. The LOD and LOQ of cotinine were 0.06 and 10 ng/mL, respectively. The LOD and LOQ of trans-3-hydroxycotinine were 0.02 and 10 ng/mL, respectively.

**Precision and accuracy**

Precision (represented by RSD) and accuracy (represented by percentage deviation) of the method results are given in Table 1. Accuracy for COT ranged from 0.98 to 5.28% and the precision ranged from 1.24 to 8.78%. Accuracy for OH-COT ranged from −2.66 to 3.72% and the precision ranged from 3.15 to 7.07%.

**Recovery**

Recoveries for the analytes ($n = 6$) are shown in Table 2. Mean recoveries for cotinine and trans-3-hydroxycotinine ranged from...
77.7 to 89.1%, and from 75.4 to 90.2%, respectively, for the three QC concentrations of 100, 1000 and 6000 ng/mL.

**Stability**

Analytes concentrations in capped GC autosampler vials were stable for up to 48 h.

**Discussion**

If some articles were published describing LC/MS methods regarding nicotine and its metabolites, only two publications described the quantification of cotinine and trans-3-hydroxycotinine in urine by GCMS method (Shulgin et al., 1992; Ji et al., 1999). The most recent article dated from 1999 and used liquid-liquid extraction procedure using volatile and...
Cotinine and trans-3-hydroxycotinine by GCMS

Table 1. Precision and accuracy for cotinine and trans-3-hydroxycotinine at three concentration levels in urine samples

| Nominal concentration (ng/mL) | Intraday | Interday |
|------------------------------|----------|----------|
|                              | Observed concentration (ng/mL) | Precision (%) | Accuracy (%) | Observed concentration (ng/mL) | Precision (%) | Accuracy (%) |
| Cotinine                     |          |          |              |              |          |              |
| 50                           | 51.1 ± 1.5 | 2.9     | 2.2          | 52.6 ± 4.5   | 8.6     | 5.3          |
| 600                          | 605.9 ± 7.5 | 1.2    | 0.9          | 622.4 ± 54.6 | 8.8     | 3.7          |
| 5000                         | 5128.3 ± 292.5 | 5.8   | 2.6          | 5155.1 ± 336.6 | 6.5    | 3.1          |
| Trans-3-hydroxycotinine     |          |          |              |              |          |              |
| 50                           | 48.7 ± 1.9 | 4.1     | -2.7         | 51.9 ± 1.7   | 3.4     | 3.7          |
| 600                          | 618.1 ± 23.9 | 3.9    | 3.0          | 589.2 ± 37.5 | 6.4     | -1.8         |
| 5000                         | 5102.9 ± 160.7 | 3.2   | 2.1          | 5125.0 ± 362.5 | 7.1    | 2.5          |

Table 2. Recoveries of cotinine and trans-3-hydroxycotinine from urine (n = 6)

| Analyte                      | Expected concentration (ng/mL) | Mean peak ratio of extracted samples | Mean peak ratio of nonextracted samples | Recovery (%) |
|------------------------------|--------------------------------|-------------------------------------|----------------------------------------|--------------|
| Cotinine                     | 100                            | 0.9                                 | 1.0                                    | 89.1         |
|                              | 1000                           | 8.8                                 | 11.4                                   | 77.7         |
|                              | 6000                           | 46.3                                | 52.5                                   | 88.2         |
| Trans-3-hydroxycotinine     | 100                            | 0.6                                 | 0.6                                    | 90.2         |
|                              | 1000                           | 4.4                                 | 5.1                                    | 87.8         |
|                              | 6000                           | 24.1                                | 32.0                                   | 75.4         |

toxic solvents as n-butyl acetate and dichloromethane (Ji et al., 1999). A recent GC method was developed for determination of urinary cotinine in active and passive smokers using liquid–liquid extraction (Malafatti et al., 2010). The sample volume of urine was high with 5.0 mL, and intra- and inter-day assay standard relative deviations were up to 14.2%. Our SPE method increased significantly the throughput and reduced the manual handling tasks associated with the liquid–liquid extraction method. However, capping SPE cartridges for use on the automated system is physically demanding and thus becomes a manual handling hazard. In Ji et al. (1999), the inter-assay CVs were 4.2–12% for OH-COT and 10–12% for COT. In our method, inter- and intra-assay were <9% for both analytes. This analytical method for the simultaneous measurement of cotinine and trans-3-hydroxycotinine in urine will be used to monitor tobacco smoking as pregnant women and will permit the usefulness of trans-3-hydroxycotinine as a specific biomarker of tobacco exposure to be determined.

Conclusion

Our report describes a rapid, sensitive, accurate and simple method for simultaneous quantification of urinary cotinine and trans-3-hydroxycotinine. SPE procedure was simplified by automating the extraction of the analytes, which are the critical steps of analysis methods. The applicability of the assay should be useful for routine monitoring of active smoking and exposure to environmental tobacco smoke. Trans-3-hydroxycotinine requires additional data to provide a useful indicator as a biomarker for tobacco smoke and to investigate differences in the metabolism of COT and OH-COT.

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