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A liquid chromatography-mass spectrometry method for nicotine and cotinine; utility in screening tobacco exposure in patients taking amiodarone

Raniah Q. Gabr[a], Marwa E. Elsherbiny[a], Vishwa Somayaji[a], P. Timothy Pollak[b] and Dion R. Brocks[a]*

ABSTRACT: A liquid chromatographic mass spectrometric (LC-MS) assay for the quantification of nicotine and cotinine in human specimens was developed. Human serum and urine (100 µL) were subjected to liquid–liquid extraction. For glucuronidated cotinine, serum was alkalinized and hydrolyzed before extraction. The dried samples were reconstituted and run using gradient flow reverse-phase liquid chromatography with MS detection. The ions utilized for quantification of nicotine, cotinine and milrinone (internal standard) were 162.8, 176.9 and 211.9 *m/z*, respectively. The mean recoveries were over 80% for cotinine and nicotine with excellent linearity between nominal concentrations and peak area ratios, over a wide concentration range. The percentage coefficient of variation and mean error of the inter- and intra-day validations were <15% for nicotine and cotinine. Analysis of serum from cardiac patients receiving amiodarone suggested that a number of patients were either active smokers or exposed to second-hand smoke. Significant concentrations of nicotine and cotinine were measured in the urine of a known smoking volunteer. The method was highly specific, sensitive and applicable as a tool in detecting and monitoring the passive exposure to tobacco smoke using small specimen volumes (0.1 mL).

Keywords: cigarette smoke; arrhythmias; tobacco; amiodarone; cotinine

Introduction

Tobacco smoke is a widely recognized public health issue that not only affects the health of those who choose to actively smoke, but also those who are in close vicinity to second-hand smoke. A pertinent example of this increased risk is provided by neonates and young children who live with a smoker. These children are at increased risk for asthma and sudden infant death syndrome (Windham *et al.*, 2000). Accurate information regarding exposure to smoke can be helpful in understanding risk and prevention of disease, and explaining current illness and treatment options (active or preventative measures). The primary means of obtaining information regarding exposure to tobacco smoke is by clinical history, but for various reasons this method is frequently unreliable (Ong *et al.*, 2005; Reeves *et al.*, 2008). One common reason is embarrassment by active smoking patients, especially amongst those suffering from illnesses related to smoking (e.g. after a myocardial infarction). In other cases, exposure may be underreported due to non-smokers considering that they are not affected by second-hand smoke exposure (e.g. when a non-smoking patient has a spouse who smokes). Given these difficulties, the assay of cigarette smoke components could potentially have utility in screening patients for exposure to tobacco for various purposes in research, and possibly in clinical applications for risk assessment or diagnostic purposes.

In the tobacco plant, nicotine (NIC) is the major alkaloid. NIC is chiral and is predominantly in the form of the *S*-(–) enantiomer, although burning the product can cause up to 11% to be converted to the *R*-(+) enantiomer (Crooks, 1999). Although NIC is the major addictive component of cigarette smoke (Balfour, 2002) its plasma half-life is too short (1–2 h) (Benowitz *et al.*, 1983) to be of practical use as a biomarker for tobacco exposure. The major oxidative metabolite of NIC is cotinine (COT), which accounts for 70–80% of NIC metabolite in plasma (Hukkanen *et al.*, 2005). COT undergoes further metabolism, particularly via C-hydroxylation, to yield *trans*-3′-hydroxycotinine and *N*-glucuronidation to yield COT-glucuronide (COT-G) and *trans*-3′-hydroxycotinine glucuronide (Benowitz *et al.*, 1994; Byrd *et al.*, 1992; Crooks, 1993). COT has a much longer elimination half life than NIC ranging from 10 to 40 h in humans (Russell and Feyerabend, 1978) and thus is considered a

* Correspondence to: D. R. Brocks, Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada. E-mail: dbrocks@pharmacy.ualberta.ca

[a] Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada

[b] The Department of Medicine, University of Calgary, Calgary, Alberta, Canada

Abbreviations used: AM, amiodarone; COT, cotinine; COT-G, COT-glucuronide; CYP, cytochrome P450; DEA, desethylamiodarone; MIL, milrinone; MTBE, methyl *tert*-butyl ether; NIC, nicotine.

suitable marker for assessing exposure to tobacco smoke. Even in non-smokers, the passive inhalation of second-hand smoke can result in detectable COT concentrations in serum (Pirkle *et al*., 1996).

There are several published methods for analysis of NIC and COT in biological specimens, including plasma, urine, hair and saliva (Eliopoulos *et al*., 1994; Hengen and Hengen, 1978; Jacob *et al*., 1981, 1991; Moyer *et al*., 2002; Shin *et al*., 2002). The most frequently used techniques involve gas chromatography or gas chromatography coupled to mass spectrometry (Hengen and Hengen, 1978; Jacob *et al*., 1981, 1991; Shin *et al*., 2002). Other methods such as enzyme-linked immunoassay and radio-immunoassay have also been reported (Knight *et al*., 1985; Van Vunakis *et al*., 1987) but have sensitivity and cross-reactivity problems. Recently, liquid chromatography coupled to mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) has been used to assay these components (Baumann *et al*., 2010; Shakleya and Huestis, 2009; Yue *et al*., 2010). Additionally, Ghosheh *et al*. (2000) succeeded in determining total COT (free COT + COT-G) in plasma indirectly using alkaline hydrolysis to liberate free COT from its conjugates prior to analysis. It was found that plasma COT levels of smokers are usually above 15 ng/mL (Pirkle *et al*., 1996).

In patients with established cardiovascular disease, exposure to tobacco smoke poses a significant threat to their health at a number of levels. In many such patients smoking is an important underlying contributing factor to their cardiovascular disease, and continued exposure is likely to worsen their clinical condition. Components of tobacco smoke can also lead to some clinically significant undesired drug–drug interactions. For example, cytochrome P450 (CYP) isoenzymes can be induced by smoke (Thum *et al*., 2006). This may have consequences on the toxicity profile of amiodarone (AM), which remains the most effective antiarrhythmic drug for a number of cardiac arrhythmias. AM is metabolized to a number of metabolites by CYP, including its major circulating metabolite, desethylamiodarone (DEA) (Elsherbiny and Brocks, 2010). This metabolite is considered to have a toxicity profile which is more severe than that of the parent drug (Bargout *et al*., 2000). For example, AM is known to cause a potentially fatal pulmonary fibrosis that is difficult to detect in some patients (Bargout *et al*., 2000; Ernawati *et al*., 2008). DEA is more toxic to pulmonary cells than AM, and patients with pulmonary toxicity tend to have increased DEA to AM serum concentration ratios (Pollak, 1999). Recent evidence has shown that CYP1A1, which can be induced in the lung by factors such as cigarette smoke, is very efficient in converting AM to DEA (Elsherbiny *et al*., 2008). Both AM and its metabolite are known to have a high uptake into lung tissues (Brien *et al*., 1987). Animal studies have suggested that AM also has an increased magnitude of pulmonary toxicity in the presence of passive exposure to tobacco smoke (Bhavsar *et al*., 2007). This knowledge provides another reason to accurately determine smoking status in patients receiving AM.

In this report a sensitive, highly specific and rapid method for the determination of total unconjugated and glucuronide-conjugated COT and NIC, using a small sample volume (100 µL), is described. To demonstrate the applicability of the method, the concentrations of NIC and COT were measured in serum of patients who had taken AM for variable lengths of time, for treatment of cardiac arrhythmias. These patients had reported being non-smokers and had been shown previously to possess relatively high ratios of plasma DEA to AM.

Experimental

(±)-NIC and (–)-COT were obtained from Sigma (St Louis, MO, USA). Apo-Milrinone injectable (Apotex, Toronto, Canada) was used as a source of milrinone (MIL) for internal standard (IS). DEA was obtained as a gift from Wyeth Ayerst (Research Monmouth Junction, NJ, USA). Acetonitrile, formic acid and water (all HPLC-grade) were purchased from (Georgetown, Ontario, Canada) and methyl *tert*-butyl ether (MTBE) from Fisher Scientific (Fair Lawn, NJ, USA). Methanol, hexane (both HPLC-grade), triethylamine and sulfuric acid (both analytical grade) were purchased from EM Science (Gibbstown, NJ, USA). Water for mobile phase was double distilled.

Chromatographic conditions

The LC-MS analysis was carried out using a single quadrupole system (Waters Micromass ZQ™ 4000 spectrometer coupled to a Waters 2795 pump and auto-sampler, Milford, MA, USA) equipped with electron spray ionization (ESI) source. The chromatographic separations of NIC, COT and IS were performed using a C_8 3.5 µm (2.1×50 mm) column as the stationary phase (Zorbax, Eclipse XDB, USA). The column was heated to 60°C. A C_8 3.5 µm (2.1×10 mm, XTerra® MS, Ireland) precolumn was placed serially proximal to the analytical column. The analytes were eluted using a mobile phase consisting of acetonitrile and 0.2% NH_4OH pumped at a flow-rate of 0.2 mL/min in a gradient manner starting at 10:90, changing to 35:65 over 10 min, then shifting back to the starting composition (10:90) over 5 min. Each of the analytes was detected using selective ion recorder (SIR) in positive ion mode. The other parameter settings were gas source temperature of 145°C, capillary voltage of 3.25 kV and cone voltages set at 24 V for analytes. The injection volume was 10 µL and gas flow of desolvation and cone gas flow were set at 550 and 50 L/h, respectively while the dissolvation temperature was 275°C. Using the apparatus, these conditions were found to be optimal for ionization of both NIC and COT and establishment of the *m/z* values of molecular ions.

Standard and stock solutions

Stock solutions of 500 µg/mL COT and NIC were prepared by dissolving 5 mg of each separately in 10 mL of water. Working solutions of both COT and NIC were prepared freshly on the day of experiment from the stocks by successive dilutions with water. Various working concentrations of 2, 10, 50, 100, 250 and 500 ng/mL in blank serum from non-smokers, and of 20, 50, 100, 500, 1000 and 5000 ng/mL in blank urine from non-smokers were prepared for generation of standard curves. An IS stock solution of 1 mg/mL was further diluted in water to prepare the working solution of 10 µg/mL. All stock solutions were protected from light and kept at 4°C until use.

Extraction procedure

To 100 µL human serum or urine was added 30 µL of IS (100 µg/mL) and 50 µL of 10 M NaOH. A total of 5 mL MTBE was then added to each tube. The tubes were then vortex mixed for 30 s and centrifuged at 3000***g*** for 3 min. The supernatant (organic layer) was transferred to clean glass tubes, and the contents acidified with 25 µL of concentrated HCl (to minimize NIC volatilization), then evaporated to dryness *in vacuo* for 35 min. The residues were reconstituted with 110 µL water and 10 µL was injected into the LC-MS system.

For total COT quantification, the 10 M NaOH (50 µL) was first added to the tubes containing 100 µL human serum or urine as in unhydrolyzed samples. The samples were heated to 70°C for 30 min in a shaking water bath to facilitate hydrolysis of conjugated species. At the end of this period, the IS (30 µL) was added and the analytes extracted into MTBE as described above.

Recovery

The serum recoveries were determined at COT concentrations of 100 and 400 ng/mL, using four replicates for each concentration. The extraction efficiency was determined by comparing the peak areas of analyte in samples to the peak areas of the same amounts of analyte directly injected to the instrument, without extraction.

Calibration, accuracy and validation

A complete validation (intra- and inter-day) assessment was performed for human serum samples. For the urine samples, one-day validation was established. Samples were prepared by adding 30 µL of IS and known amounts of COT and NIC to 100 µL human matrix, providing a concentration range of 2–500 ng/mL for both analytes in the case of serum and 20–5000 ng/mL in the case of urine. The ratios of COT or NIC to the respective IS peak areas were calculated and plotted vs nominal COT or NIC concentrations to construct calibration curves. Data for calibration curves were weighted by a factor of 1/concentration.

Intraday accuracy and precision of the assay were determined using ascending concentrations of COT and NIC in both matrices. For both analytes the selected concentrations were 2, 10, 100 and 500 ng/mL in serum (five replicates per concentration), or 20, 100, 1000 and 5000 ng/mL in urine (four replicates per concentrations). Regarding human serum samples, to permit the assessment of inter-day accuracy and precision, the assay was repeated on three separate days. For each daily run, a set of calibration samples separate from the validation samples was prepared to permit quantification of the peak area ratios of both COT and NIC to internal standard. Precision and accuracy were assessed using percentage coefficient of variation (CV%) and mean intra- or inter-day percentage error, respectively. As for urine, a partial (one day) validation was used with a replicate of five samples for each concentration.

The peaks were assessed for symmetry using the symmetry index. The peak width at 10% of peak height was first determined. The latter part of the width from time of peak height onwards was then divided by the first part of the width up to the time of peak height.

Assessment of matrix effect

To determine the effect of serum and urine on the process of ionization of the analytes, NIC, COT and IS, we applied the post-extraction spike method proposed by Matuszewski *et al.* (Matuszewski, 2006; Matuszewski *et al.*, 2003). Two six-replicate sets of analyte-free urine or plasma matrices, respectively, were extracted and dried. In a third set, the same volume of HPLC water was subjected to extraction. After the extracts were dried, 110 µL of water containing NIC (20 ng/mL), COT (20 ng/mL) and IS (3000 ng/mL) was added to the tubes. The samples were injected into the LC-MS and responses recorded. The responses of the analytes in the biomatrix-containing extracts were compared with those of the samples only containing water.

Applicability of the assay

Patients attending an arrhythmia clinic provided written informed consent for collection of samples and historical information to be used for research into improvements in the use of amiodarone. Sera from 19 of those patients who had been receiving AM with documented elevations in their ratio of DEA to AM was subjected to assay of NIC, total COT, COT, AM and DEA. An established liquid chromatography and mass spectrometry method was used for the simultaneous assay of AM and DEA (Shayeganpour *et al.*, 2007). The assay had a validated lower limit of quantitation of 2.5 ng/mL for each of AM and DEA, based on 100 µL of serum.

Results

The molecular ions of IS, COT and NIC were represented by peaks with *m/z* of 211.9, 176.9 and 162.8 respectively (Fig. 1). The retention times of eluted IS, COT and NIC were approximately 1.8, 3.5 and 7.11 min, respectively (Fig. 2). Each peak had no interference from endogenous compounds in serum or urine. The peaks were symmetrical, with symmetry indices of 1.1 being observed for NIC, COT and IS. The average extraction recoveries in serum were 84.0 and 89.3% for 100 and 400 ng/mL of COT, respectively. The average extraction recoveries in serum for NIC were 87.1 and 80.3% for 100 and 400 ng/mL, respectively. There were excellent linear relationships between the peak area ratios concentrations of both analytes over the range of 2–500 and 20–5000 ng/mL in serum and urine, respectively. A representative standard curve in serum using peak area ratios yielded slopes of 0.025005 and 0.014969 for NIC and COT, respectively. Corresponding intercepts were 0.03365 and 0.056397, respectively. The mean r^2 for the three standard curves was 0.9984 for both NIC and COT. As for the urine analysis, standard curves based on area ratios yielded slopes of 0.0236 and 0.0234 for NIC and COT respectively. Corresponding intercepts were 0.139 and 0.589 respectively.

The assay was tested for its comparative ability to measure NIC and COT in plasma and serum spiked with 200 ng/mL of each analyte (n=4 tubes each of serum and plasma). The measured analyte concentrations were essentially the same (<6.5% difference) for both analytes in serum and plasma. Regarding the matrix effect, there was no significant matrix effect on the ionization of COT and IS. However, both matrices were shown to have a response enhancement (by 30%) on NIC.

The assay CVs for both the intra-and inter-day assessments were equal to or less than 15% (Table 1). Mean inter-day error in human serum was less than 9% for both NIC and COT while it was up to 14% for both analytes in urine (Table 2). Based on the inter- and intra-day coefficient of variation and mean error, which were much less than 20%, it could be claimed, based on 0.1 mL of specimen, that the lower limits of quantitation (LLQ) of both NIC and COT were 2 ng/mL in serum and 20 ng/mL in urine.

In the 19 cardiac patients receiving AM, representing a total number of 21 visits to the clinic, measurable concentrations of total COT and NIC were measured in seven and two patient serum samples, respectively (Table 3). Small peaks below the LLQ of COT were observed in most of the other serum samples. There was a wide range of DEA to AM serum concentration ratios, and no significant difference in the ratio between those with and without measurable concentrations of total serum COT (Table 3, Fig. 3). Patients with the lowest concentrations of serum AM tended to have the highest DEA: AM ratios (Fig. 3).

Two patients had repeat samples available, which were subjected to analysis (Table 3). In the initial sample drawn in patient 2, serum COT was measurable at levels suggestive of passive exposure to smoke, or light smoking. In the second sample drawn almost 14 months later, COT was not detected in the serum, but large increases in the AM and DEA concentrations were observed indicative of significant accumulation of the drug. In the other repeated patient (patient 16), the samples were spaced 9 months apart. There was no evidence of exposure to tobacco in either sample, and little accumulation of DEA or AM. The timing of onset of AM therapy was not recorded so it was not possible to draw conclusions regarding spaced sample analysis of AM and accumulation. This method was also applied to measure the urine concentrations of NIC and COT in a smoking subject, which were found to be 206 and 843 ng/mL, respectively.

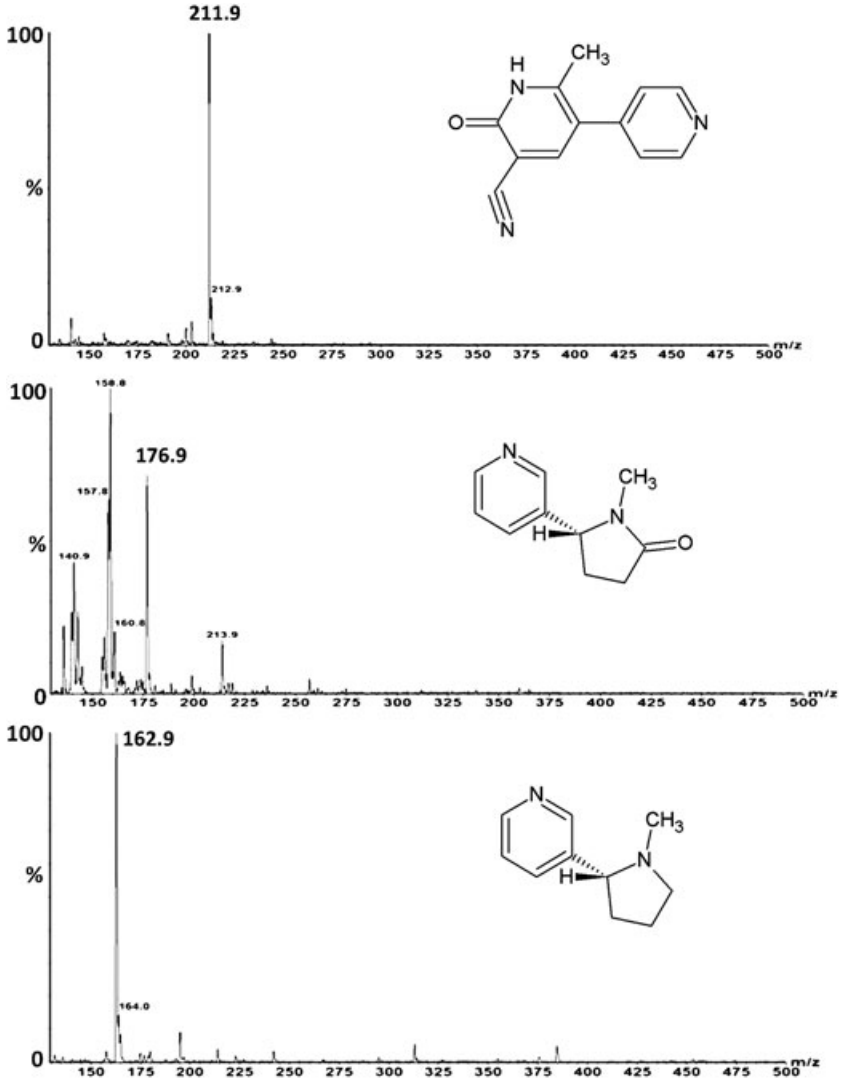

Figure 1. Mass spectra and chemical structures of the internal standard, milrinone (upper panel); cotinine (middle panel); and nicotine (lower panel).

Discussion

A sensitive and robust LC-MS assay with liquid–liquid extraction sample preparation has been developed for quantification of NIC and total COT in human serum and urine. This method offered an improved selectivity and sensitivity over traditional HPLC assay methods, with validated lower limits of quantitation of 2 ng/mL in serum and 20 ng/mL in urine, using only small volumes (100 µL) of serum or urine. Although NIC and COT are chiral, we adopted a non-stereospecific approach because our aim was to use the assay as a screening tool for tobacco exposure. As such, sensitivity and specificity were more important issues than the ability to separate enantiomers.

Most of the available LC-MS/MS methods for analysis of NIC and COT use solid-phase extraction for sample preparation (Shakleya and Huestis, 2009; Yue *et al*., 2010). Although such extraction methods are very useful, they require the use of disposable columns which can add expense to the method. Recently, Baumann and his colleagues successfully established an LC-MS method that has LLQ for NIC and COT assay in human serum of 2 and 5 ng/mL, respectively, but this was based on 0.5 mL of specimen (Baumann *et al*., 2010). The reported method yielded approximately 70% recovery for COT using solid-phase extraction, and it was suggested that the use of liquid–liquid extraction would decrease the recovery. In contrast, use of the liquid–liquid extraction method herein described could yield recoveries of over 84% at concentrations equal to or below 100 ng/mL of COT. It was also established that the current method could be adapted to indirectly measure the amount of COT that underwent glucuronidation. Alkaline hydrolysis of COT-G has been used by Ghosheh *et al*. (2000) to determine total COT in serum of smokers using 0.5 mL of specimen. An HPLC-UV method with a retention time for COT of 20 min was used. In the present method, heating of the samples to facilitate glucuronide hydrolysis added no interfering chromatographic peaks in the analysis (Fig. 2). The measurement of the sum of COT and NIC and all associated metabolites in serum has been proposed to be a good measure of NIC exposure for pharmacoepidemiological purposes (de Leon *et al*., 2002). It is to be noted that COT

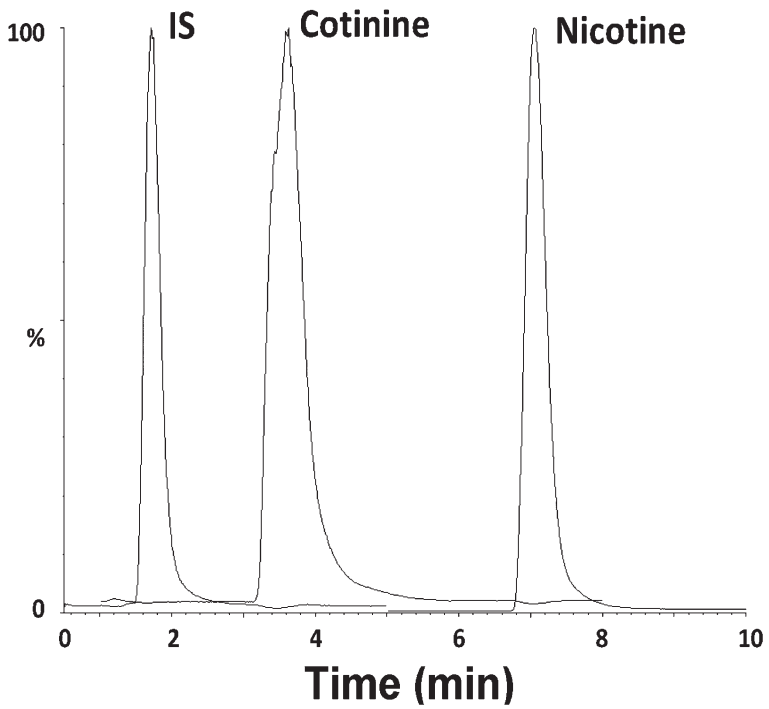

Figure 2. SIR chromatograms of a representative human serum sample.

Table 1. Intra- and inter-day validation data for the assay of nicotine and cotinine in human serum ($n = 5$)

		Intraday			Interday		
Expected concentration, ng/mL	Analyte	Mean ± SD (CV%), ng/mL			Mean ± SD, ng/mL	CV%	Error %
2	Nicotine	2.21±0.27 (10.3)	1.90±0.20 (10.4)	2.09±0.31 (15.0)	2.07±0.16	7.56	3.33
	Cotinine	2.00±0.21 (10.2)	1.72±0.18 (10.3)	1.63±0.10 (6.03)	1.78±0.19	10.8	-10.8
10	Nicotine	11.1±0.41 (3.69)	11.6±0.34 (2.40)	9.12±1.10 (12.1)	10.6±1.32	12.5	6.13
	Cotinine	10.8±0.70 (6.51)	11.3±0.44 (3.91)	10.6±0.72 (6.79)	10.9±0.37	3.49	8.90
100	Nicotine	93.2±4.62 (4.96)	113±10.3 (9.11)	96.7±4.26 (4.40)	101±10.8	10.7	1.08
	Cotinine	107±2.49 (2.33)	105±4.91 (4.67)	108±6.49 (6.04)	107±1.30	1.22	6.50
500	Nicotine	528±7.48 (1.42)	522±34.9 (6.68)	464±25.9 (5.58)	505±35.3	7.00	0.976
	Cotinine	536±16.2 (3.03)	494±29.0 (5.87)	497±8.70 (1.75)	509±23.6	4.64	1.82

Table 2. One day validation data for the assay of nicotine and cotinine in human urine ($n = 4$)

Expected concentration, ng/mL	Analyte	Mean ± SD, ng/mL	CV%	Mean error %
20	Nicotine	18.7±1.92	10.3	−6.69
	Cotinine	22.2±0.91	4.11	11.0
100	Nicotine	97.3±12.8	13.1	−2.67
	Cotinine	103±7.50	7.31	2.63
1000	Nicotine	1139±70.9	6.22	13.9
	Cotinine	1016±34.2	3.36	1.64
5000	Nicotine	4518±333	7.37	−9.65
	Cotinine	4912±215	4.55	−5.74

ionization, which is the primary analyte of interest, was not affected by either plasma or urine.

Because of its significance in cardiac disease, cardiologists strive for accuracy in documentation of smoking history. The assay of COT in serum could provide a better judge of exposure (Malkawi *et al.*, 2009). Closet smoking is common in hospitalized patients, and was found to be between 8 and 32% in patients admitted to hospital, many of whom deny smoking. The

Table 3. Concentrations (ng/mL) of total cotinine (total COT), cotinine (COT), cotinine glucuronide (COT-G), nicotine (NIC), amiodarone (AM) and desethylamiodarone (DEA) in cardiac patients. Ratios of DEA:AM in each of the samples are also provided

Patient	Total COT	COT	COT-G	NIC	DEA	AM	DEA:AM
1	2.56	<2	NC	<2	1757	957	1.84
2a	4.19	<2	NC	<2	1783	2253	0.79
3	4.71	<2	NC	<2	1120	1428	0.78
4	5.23	<2	NC	<2	1223	1007	1.21
5	8.22	<2	NC	<2	184	144	1.28
6	23.4	10.7	12.7	<2	2161	1428	1.51
7	625	267	358	4.8	1021	778	1.31
2b	<2	<2	NC	<2	5370	2993	1.79
8	<2	<2	NC	<2	1779	1669	1.07
9	<2	<2	NC	<2	1479	1687	0.88
10	<2	<2	NC	2.2	1622	2229	0.73
11	<2	<2	NC	<2	279	76	3.66
12	<2	<2	NC	<2	810	268	3.02
13	<2	<2	NC	<2	1620	1428	1.13
14	<2	<2	NC	<2	768	1073	0.72
15	<2	<2	NC	<2	323	74	4.38
16a	<2	<2	NC	<2	1173	831	1.41
16b	<2	<2	NC	<2	2417	3192	0.76
17	<2	<2	NC	<2	637	450	1.42
18	<2	<2	NC	<2	1269	1082	1.17
19	<2	<2	NC	<2	156	106	1.47
					Samples with measurable cotinine		
					1321±651	1142±656	1.25±0.38
					Samples without measurable cotinine		
					1407±1313	1226±1040	1.69±1.16

NC, not calculable due to lack of quantifiable unchanged COT. Where total COT was measurable, in those samples it is likely that the majority of the concentration is present as COT-G.

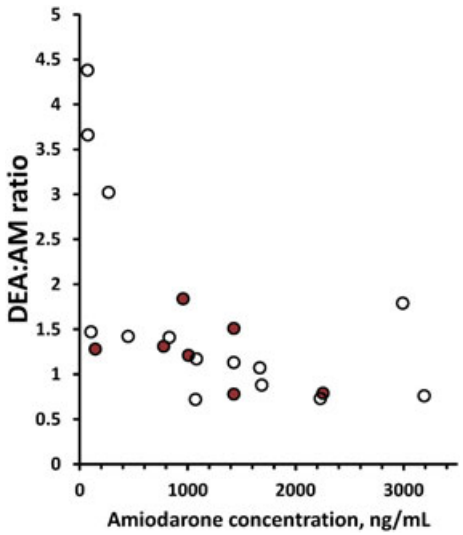

Figure 3. The relationship between amiodarone serum concentrations and the corresponding ratios of desethylamiodarone to amiodarone. Shaded symbols represent patient samples where total cotinine (sum of glucuronidated and unchanged cotinine) was measured at concentrations above the lower limit of quantitation of the assay (2 ng/mL).

applicability of the current method was demonstrated in its ability to measure COT and NIC in serum of cardiac patients. The current findings appear to be in agreement with previous reports. Of the 19 purportedly non-smoking patients, seven (37%) were found to possess significant levels of serum COT, providing objective evidence of exposure to tobacco. Of these, two patients showed clear evidence of active smoking, with total COT concentrations exceeding 10 ng/mL. Five patients had detectable total COT serum concentrations ranging from 2 to 10 ng/mL, suggesting either light smoking or second-hand exposure to smoke. Although the limit of detection of the assay was 2 ng/mL, it is of interest that peaks representing COT were seen in several other patients, albeit at levels below the validated lower limit of detection. NIC peaks were hardly detectable in patient sera, probably due to its short elimination half-life, but were high in the urine of a smoking subject. It is difficult to make any conclusion regarding the relationship between COT serum concentrations and the DEA:AM ratio because the patients differed in cumulative AM dose, and drug and metabolite accumulation is known to be both extensive and prolonged (Brocks and Mehvar, 2010). Nevertheless, it is possible that, for at least some of these patients (Fig. 3), the elevated DEA:AM ratios could be in part attributed to exposure to tobacco smoke, components of which are known to induce the metabolism of AM to DEA (Elsherbiny and Brocks, 2010; Elsherbiny *et al*., 2010).

Conclusions

The reported assay method showed a high calibre of linearity, sensitivity, reliability and precision, with a lower limit of

quantitation of 2 ng/mL in human serum, and 20 ng/mL in urine, for both NIC and COT. The method was applied as a tool for the detection and monitoring of the exposure to tobacco smoke wherein a significant number of cardiac patients taking AM were found to have been exposed to tobacco. The finding of significant concentrations of COT in the serum of several cardiac patients illustrates the need for caution in viewing patient records regarding recent smoking history and the need for diligence in monitoring such patients receiving AM, a drug with potential pulmonary toxic effects.

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