Cotinine is the main metabolite of nicotine and is used as an indicator of exposure to tobacco smoke. A method has been developed for quantification of cotinine in pericardial fluid and whole blood collected from autopsy casework involving cases of infant death. Sample clean-up was achieved by solid-phase extraction with a mixed-mode column. Cotinine was quantified by liquid chromatography–tandem mass spectrometry. Positive ionization was performed in the multiple reaction monitoring mode. Two transitions were monitored for the analyte and one for the internal standard, cotinine-d$_3$. The calibration range was 0.9–176 ng/mL for cotinine in both matrixes. The recovery of the analyte ranged from 86 to 92%, and the between-assay precisions ranged from 4 to 6% relative standard deviation. Whole blood and pericardial fluid samples from 95 infant deaths obtained during autopsy were analyzed. A strong correlation ($R^2 = 0.97$) was found between the cotinine concentrations in pericardial fluid and blood. The correlation was not affected by the postmortem time interval. This study demonstrates that pericardial fluid may be an alternative specimen to blood for quantification of cotinine in forensic autopsies.

**Abstract**

Cotinine is the main metabolite of nicotine and is used as an indicator of exposure to tobacco smoke. A method has been developed for quantification of cotinine in pericardial fluid and whole blood collected from autopsy casework involving cases of infant death. Sample clean-up was achieved by solid-phase extraction with a mixed-mode column. Cotinine was quantified by liquid chromatography–tandem mass spectrometry. Positive ionization was performed in the multiple reaction monitoring mode. Two transitions were monitored for the analyte and one for the internal standard, cotinine-d$_3$. The calibration range was 0.9–176 ng/mL for cotinine in both matrixes. The recovery of the analyte ranged from 86 to 92%, and the between-assay precisions ranged from 4 to 6% relative standard deviation. Whole blood and pericardial fluid samples from 95 infant deaths obtained during autopsy were analyzed. A strong correlation ($R^2 = 0.97$) was found between the cotinine concentrations in pericardial fluid and blood. The correlation was not affected by the postmortem time interval. This study demonstrates that pericardial fluid may be an alternative specimen to blood for quantification of cotinine in forensic autopsies.

**Introduction**

Nicotine is the main psychoactive compound in cigarette smoke. One of the major metabolites of nicotine is cotinine. Cotinine is slowly cleared from the body with a half-life of ~20 h, which is approximately 10 times longer than the half-life of nicotine (1,2). Cotinine has been determined in a number of body fluids including plasma, urine, meconium, and saliva (3–9). Thus, cotinine is useful as a biomarker for estimating both active and passive exposure to tobacco smoke (10). Cigarette smoke has a well-documented negative influence on health. With regard to sudden infant death syndrome (SIDS), prenatal exposure to tobacco smoke is the most important environmental risk factor next to the prone sleeping position (11,12). Recent evidence suggests that postnatal exposure of tobacco smoke in the period prior to death is also associated with sudden death in infancy (13–15). Consequently, cotinine measurements in forensic samples, especially from children, could be valuable in determining tobacco exposure with possible implications for the manner of death. As the amount of whole blood and urine collected in postmortem investigations of infants may be limited, pericardial fluid represents an alternative sample matrix. Pericardial fluid has previously been shown to be a useful matrix for determination of cotinine by gas chromatography–mass spectrometry (GC–MS) (13,14).

Milerad et al. (13) reported a cut-off point at 5 ng/mL cotinine to identify infants who had been significantly exposed in the last few hours of life. Bajanowski et al. (14) found that the cotinine levels in pericardial fluid from SIDS victims corresponded to the reported number of cigarettes smoked per day by the caretaker.

In order to enable assessment of cotinine levels in samples from infant deaths, the aim of the present study was, firstly, to develop a sensitive, robust, and specific liquid chromatography (LC)–tandem MS method, and secondly, to evaluate the relationship between pericardial fluid and whole blood with regards to cotinine concentrations.

**Material and Methods**

**Chemicals and reagents**

Cotinine was obtained from Cerilliant (Austin, TX) and Alfa Aesar (Karlsruhe, Germany). Cotinine-d$_3$ was purchased from Cerilliant. Other chemicals were of high-performance liquid chromatography (HPLC) or analytical grade from various commercial sources. The Oasis MCX (60 mg, 3 mL) extraction columns were purchased from Waters (Milford, MA).
Standard solutions
Two separate stock solutions were prepared to a concentra-
tion of 170 µg/mL and 1200 µg/mL in methanol and were used
for calibrator and quality control (QC) samples, respectively.
Aqueous calibration solutions with concentrations of 8.8, 17.6,
88.1, 176.2, 881, and 1762 ng/mL were prepared from the
stock solution. Aqueous QC solutions were prepared from the
stock solution to concentrations of 13.4, 97.6, and 723.3
ng/mL. The internal standard cotinine-d$_3$ was diluted with
water to a concentration of 3.6 µg/mL. The stock and aqueous
solutions were stored at –20°C and 4°C, respectively.

Whole blood. Spiked calibration and QC samples were prepared
by adding 50 µL of aqueous calibration and QC solutions
to 0.5 mL blank sodium fluoride whole blood from healthy
donors (The Blood Centre at Ullevaal University Hospital, Oslo,
Norway).

Pericardial fluid. Because of the poor availability of blank
pericardial fluid, calibration and QC samples were prepared by
adding 100 µL of aqueous calibration and QC solutions to 1 mL
detonized water. In addition, a batch of pericardial fluid from
different donors ($n = 6$, Institute of Forensic Medicine, Oslo,
Norway) was collected at autopsy and pooled without knowl-
dge of smoking status. The batch was used to examine the be-
tween-assay precision of the method, and a blank pericardial
fluid sample from one donor was used to examine the recovery
of the method.

Specimens
During autopsy, samples of pericardial fluid were collected
from the pericardial sac and samples of whole blood were col-
clected from the heart and/or great vessels by suction syringe.
Samples from a total of 95 cases of infant deaths were ob-
tained during autopsy between 1990 and 2004 at the Institute
of Forensic Medicine and stored at –80°C until analysis. The
median range of time interval between death and autopsy was
25 h (5–97 h). The pericardial samples were divided into two
groups based on the visible color of the fluid. Forty-six samples
had a distinct yellow appearance, whereas 49 cases appeared to
be contaminated with blood because postmortem blood sam-
plies had been collected prior to the forensic autopsy.

Sample treatment
To 0.5 mL whole blood, 50 µL cotinine-d$_3$ was added. The
mixed blood sample was precipitated with 1.8 mL ice-cold ace-
tonitrile, centrifuged at 2200 × g for 2 min, and placed in
-freezer (–20°C) for 30 min. The supernatant was applied to an
HPLC column. The column was washed with water (2 mL),
acetonitrile/water (5:95, v/v), centrifuged at 2200 × g for 2 min,
and then equilibrated with 0.1 M hydrochloric acid (1 mL),
and methanol (2 mL). The analyte was eluted with 2 mL dichloromethane/iso-
-propanol/ammonium hydroxide (80:20:2, v/v). The eluates were
evaporated to dryness under $N_2$ (40°C) and dissolved in 100 µL
acetonitrile/water (5:95, v/v).

Pericardial fluid (1 mL) was prepared by addition of 100 µL
cotinine-d$_3$ prior to solid-phase extraction (SPE). The same
extraction procedure as described previously was used for peri-
cardial fluid samples. Samples contaminated with blood during
autopsy were centrifuged (1215 × g for 2 min) prior to the ex-
traction to avoid blockage of the extraction column.

Instruments
HPLC. LC was performed using a Waters Alliance 2695
system (Manchester, U.K.). Separation was performed on a Wa-
ters Atlantis dC18 (2.1 × 50 mm, 3.0 µm) column, using gra-
dient elution at a flow rate of 0.3 mL/min with the following
solvent system: 5 mM ammonium acetate, pH 5 (A) and 100%
acetonitrile (B). The system was run in a linear gradient from
5% B to 20% B for 3 min, increased up to 90% B for 2 min, and
held for 1 min. Re-equilibration of the HPLC column was
achieved as the start conditions were held for 1.5 min before
the next injection. The column temperature was held at 35°C
during analysis. The injection volume was 10 µL.

MS–MS. A Quattro Ultima Pt tandem-quadrupole MS (Wa-
ters) equipped with a Z-spray electrospray interface was used.
Positive ionization was performed in the multiple reaction
monitoring (MRM) mode. The capillary voltage was set to 1.0
kV, the source block temperature was 120°C, and the desolva-
gation gas (nitrogen) was heated to 400°C and delivered at a flow
rate of 400 L/h. The cone gas (nitrogen) was set to 60 L/h and
the collision gas (argon) pressure was maintained at 0.5 psi.
The $m/z$ 177.0 → 80.1 and $m/z$ 177.0 → 98.1 transitions (cone
voltages: 40 V, collision energy: 15 eV) were monitored for co-
tinine, and $m/z$ 180.1 → 80.1 transition (cone voltages: 50 V,
collision energy: 15 eV) for cotinine-d$_3$. System operation and
data acquisition were controlled using Mass Lynx 4.0 software
(Waters). All data were processed with the QuanLynx quantifi-
cation program (Waters).

Identification and quantification
The analyte was identified by comparing the retention time
of the respective MRM transitions with the retention time of
the corresponding calibrator and QC samples. The ratio be-
tween the two MRM transitions for cotinine was also com-
pared with those of the corresponding calibrator and QC sam-
ples, and should not deviate more than 10% from the average
ratio.

The six-point calibration curves were based on peak-height
ratios of the analyte relative to the internal standard using a
weighted (1/x) second-order regression line that included the
origin.

Method validation
Within-assay precision was estimated by analysis of 10 sep-
ate replicates of QC samples at three concentration levels in
a single assay ($n = 10$). Between-assay precision and accuracy
were determined by analysis of aliquots of each QC concentra-
tion at 10 different days, 1 replicate in each assay.

Whole blood. Limits of quantification (LOQ) were deter-
mimed by analyzing 5 different drug negative whole blood spec-
imens on 10 successive days, 1 replicate on each day. LOQ was
defined as the analyte concentration giving an MRM transition
($m/z$ 177.0 → 80.1) signal height equal to that of the mean of
the signal of the negative samples plus 10 standard deviations
of these samples. The recoveries (total and SPE recovery) of
whole blood were determined with six replicates at each QC
concentration. Total recovery was determined by measuring

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peak heights obtained when the analytes were added before sample precipitation and extraction. SPE recovery was estimated by measuring peak heights obtained when the analyte was added to the supernatant of the precipitated whole blood sample. Recoveries were calculated by comparing peak heights with those obtained when the analytes were added after sample precipitation and extraction. In all cases, the internal standard was added after sample preparation. Matrix effects (ME) were evaluated in whole blood by the method proposed by Matyszewski et al. (16). The analyte signal in the spiked mobile phase was compared with the analyte signal in the matrix fortified after extraction, and the ME was defined as ME% = (extracted matrix height/mobile phase height) × 100. Five replicates of mobile phase and five replicates of whole blood (from five different individuals) extracts were analyzed. The concentrations corresponded to the lowest and highest QC sample.

Pericardial fluid. A batch of mixed pericardial fluid sample collected from several donors was analyzed on 10 successive days, 1 replicate on each day, to determine the between-assay precision. The concentration of cotinine was determined to be 115 ng/mL. For the pericardial fluid method, the lowest calibration sample was fortified with acetonitrile/water (5:95, v/v) at a concentration to 0.35 ng/mL to confirm that the signal-to-noise at the determined LOQ values was greater than 10:1. The SPE recovery of cotinine in water was determined at each QC concentration with six replicates and was performed by the same procedure as whole blood. In addition, the SPE recovery of a blank pericardial fluid sample was assessed at the lowest QC sample (n = 6).

Results and Discussion

Method validation

Whole blood. The MRM chromatograms of blank whole blood spiked with analyte and internal standard at the level of the lowest calibrator are shown in Figure 1. The within-assay

![Figure 1. MRM chromatograms of cotinine (0.9 ng/mL) in whole blood from the lowest calibrator sample.](https://academic.oup.com/jat/article-abstract/33/4/218/774673)

<table>
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<tr>
<th>Table I. Calibration Range, LOQ, Within-Assay Precision, Between-Assay Precision, Bias, and Recovery for Whole Blood</th>
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<td><strong>Analyte</strong></td>
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<td>Cotinine</td>
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* Control samples in water.

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<th>Table II. Calibration Range, LOQ, Within-Assay Precision, Between-Assay Precision, Bias, and Recovery for Pericardial Fluid</th>
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precision, between-assay precision, bias, recovery, and LOQ for the analyte are presented in Table I. The within-assay relative standard deviations (RSDs) were 3.2%–4.9%, and the between-assay RSDs were 4.2%–5.5%. The bias was in the range –13.6 to –8.9%, and recoveries ranged from 94 to 97%.

The ME % displayed a range from 95 to 97%, and RSDs for the peak height of cotinine in set 1 were between 2.9 and 3.3%, and for set 2, between 5.5 and 7.3%. The higher variability in set 2 than in set 1 indicated an ME. The RSDs for set 2 using the peak-height ratio of cotinine and internal standard showed improved RSDs (3.3–4%) when compared to no correction with internal standard. The results indicate that the internal standard had a compensatory effect both on the precision and reliability of the quantification of cotinine. The observed ME was considered to be acceptable.

**Pericardial fluid.** Calibration and QC samples in water were mainly utilized for the validation of the method and the ME was not evaluated. The within-assay precision, between-assay precision, bias, recovery, and LOQ for the analytes are presented in Table II. The within-assay RSDs were 1.9–4.7%, and the between-assay RSDs were 3.6–6.4%. The bias was in the range of –13.1 to –7.2%, and recoveries ranged from 87 to 90%. The RSD of the between-assay precision in a real pericardial fluid sample (at 115 ng/mL) was 5.3%. Figure 2 presents the MRM chromatogram of an authentic pericardial fluid sample.

**Application**

Samples from 95 infant deaths were analyzed. The median and range of cotinine concentrations in whole blood and pericardial fluid were 2.8 ng/mL (0–66.8) and 3.0 ng/mL (0–66.8), respectively. Thirty-six (36%) of the 95 cases had a cotinine level in pericardial fluid exceeding 5 ng/mL, indicating a significant exposure prior to death (13). Full presentation of the clinical dataset will be presented in a subsequent paper.

Linear regression analysis (SPSS 16.0) was used to compare the cotinine levels within the two samples. A strong correlation between pericardial fluid and whole blood was found ($R^2 = 0.97, P < 0.001$, Figure 3). Interestingly, similar results have been obtained in a correlation study between blood and pericardial fluid samples for a number of neutral, basic, and acidic drugs (17). In addition, a strong correlation between pericardial fluid and blood concentrations for benzoylecgonine was also demonstrated by Contreras et al. (18).

In the present study, the correlation between cotinine measured...
measurements in whole blood and pericardial fluid was not affected by the time interval between death and autopsy (Figure 4). The pericardial fluid samples were to different extents contaminated with blood. At the hospital, a blood sample of the heart is routinely taken before autopsy and will in some cases consequently contaminate the pericardial fluid sample. The pericardial samples were divided into two groups: pure pericardial fluid (n = 46) and pericardial fluid contaminated with blood (n = 49). There was no significant difference between those two groups regarding the correlation coefficients of the regression lines, $R^2 = 0.99$ and 0.97, respectively.

**Conclusions**

The LC–MS–MS method proved to be robust and specific for the determination of cotinine in both blood and pericardial fluid. Pericardial fluid may be an alternative matrix to blood for cotinine analysis in forensic autopsies, as a strong correlation between the cotinine concentrations in the two matrices was demonstrated. This paper is a part of a larger study on nicotine exposure and environmental risk factors for SIDS.

**References**