Determination of Cocaine, Benzoylecgonine, Cocaethylene, and Norcocaine in Human Hair Using Solid-Phase Extraction and Liquid Chromatography with Tandem Mass Spectrometric Detection

Application

Forensics

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Abstract

A quantitative analytical procedure for the determination of cocaine, benzoylecgonine, cocaethylene, and norcocaine in hair has been developed and validated. The hair samples were washed, incubated, and any drugs present were quantified using mixed-mode solid-phase extraction and liquid chromatography with tandem mass spectrometric detection in positive atmospheric pressure chemical ionization mode. For confirmation, two transitions were monitored and one ion ratio was determined, which was within 20% of that of the known calibration standards. The monitoring of the qualifying transition and requirement for its presence within a specific ratio to the primary ion limited the sensitivity of the assay, particularly for benzoylecgonine; however, the additional confidence in the final result as well as forensic defensibility were considered to be of greater importance. Even with simultaneous monitoring, the concentrations proposed by the United States federal guidelines for hair analysis were achieved. The limits of quantitation were 50 pg/mg; the limit of detection was 25 pg/mg. The intra-day precision of the assays at 100 pg/mg (n = 5) was 1.3%, 8.1%, 0.8%, and 0.4%; inter-day precision 4.8%, 9.2%, 15.7%, and 12.6% (n = 10) for cocaine, benzoylecgonine, cocaethylene, and norcocaine, respectively. The methods were applied to both proficiency specimens and to samples obtained during research studies in the USA.

Introduction

Cocaine (COC) and its metabolites are included in the proposed United States federal regulations for hair analysis. The suggested cut-off concentration for the metabolites is 50 pg/mg, which is difficult to achieve routinely using electron impact gas chromatography-mass spectrometry (GC/MS) [1,2]. This may be due to the inability to derivatize cocaethylene (CE) to improve its response; the coelution of norcocaine (NC) and CE, or potentially similar ions for the derivatives of NC and benzoylecgonine (BZE). Procedures have been developed to approach the proposed detection requirements, including positive chemical ionization GC/MS [3], and GC with tandem mass spectrometry [4].

There are two publications describing the analysis of COC and its metabolites in hair using LC/MS/MS in atmospheric pressure chemical ionization (APCI) mode, in a similar manner to our approach [5,6]. The first of these analyzes only COC and BZE, but more importantly, both procedures monitor only one transition in the multiple reaction-monitoring mode (MRM). Recently, several authors have focused on the need for the monitoring of a second transition, allowing the ratio between the abundance of the primary and secondary ions to be calculated and establishing more

confidence in the final result. Maralikova and Weinmann [7] note that guidelines for confirmatory analysis using LC/MS/MS have not yet been established and suggest that the monitoring of at least two transitions is required to provide sufficient identification of drugs. Johansen and Bhatia [8] describe the analysis of COC and its metabolites in whole blood and urine using LC/MS/MS, focusing on the establishment of identification criteria based on two MRM transitions, their ratio, and retention time. This is particularly important in assays that include compounds with similar molecular weights and chemical properties, since the same product ion is often present.

Using these suggestions for tandem mass spectrometry, we developed and validated a procedure using LC/MS/MS for the analysis of COC and its metabolites in hair in order to provide additional confidence in the generated result. The method was applied to specimens received by our laboratory from proficiency programs and research studies. Structures of the compounds are shown in Figure 1.

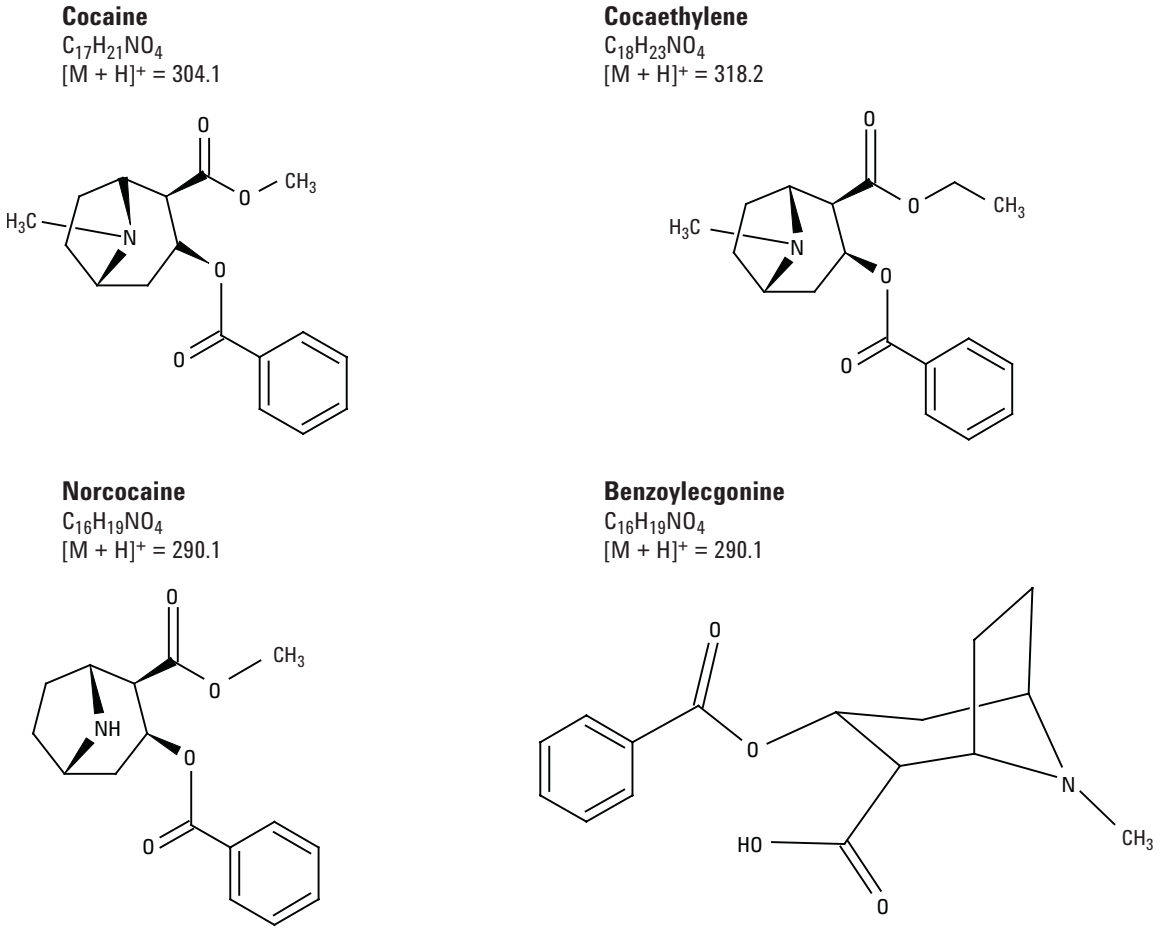

Figure 1. Structures of cocaine and metabolites analyzed in this work.

Experimental

Sample Preparation

Standards and Reagents

Deuterated internal standards (BZE-d3, COC-d3, NC-d3, and CE-d8) as well as unlabeled drug standards for each of the drugs were obtained from Cerilliant (Round Rock, TX). Solid-phase extraction columns (Clin II, 691-0353T) were obtained from SPEWare, (San Pedro, CA). All solvents were HPLC grade or better, and all chemicals were ACS grade.

Calibrators

For the chromatographic calibration standards, a working solution containing deuterated internal standards was prepared in methanol at a concentration of 200 ng/mL. Unlabeled drug standards were prepared in methanol at the same concentration. All the working solutions were stored at –20 °C when not in use. For each batch, eight calibration standards were prepared in drug-free hair (10 mg). Drug concentrations of 25, 50, 100, 200, 500, 1,000, 2,000 and 10,000 pg/mg of hair were prepared (internal standard concentration: 1,000 pg/mg).

Sample Preparation for Chromatographic Analysis

An aliquot of hair (10 mg) was briefly rinsed with methylene chloride (1.5 mL) to remove hair treatments such as mousse, spray, gels, etc., and allowed to dry. The hair was cut into small pieces and internal standard was added (50 µL). 0.025 M phosphate buffer (pH 2.7; 1.5 mL) was added and the hair was sonicated at 75 °C for 2 hours. The buffer was decanted into clean glass tubes and 0.1 M sodium phosphate buffer (pH 6.0; 1 mL) was added to each calibrator, control, or hair specimen. The mix was centrifuged for 10 min to ensure that no hair strands were applied to the solid-phase extraction column. Solid-phase mixed-mode extraction columns (Clin II, 691-0353T) were placed into a positive pressure manifold. Each column was conditioned with methylene chloride: methanol: ammonium hydroxide (78:20:2 v,v 2 mL), ethyl acetate (2 mL), methanol (2 mL), and 0.1 M hydrochloric acid (1 mL). The samples were allowed to flow through the columns, and then the columns were washed with deionized water (2 mL), 0.1 M hydrochloric acid (2 mL), methanol (2 mL), and ethyl acetate (2 mL). The columns were allowed to dry between washes under nitrogen pressure (30 psi; 2 min). The drugs were finally eluted using freshly prepared methylene chloride: methanol: ammonium hydroxide (78:20:2 v,v 3 mL). The extracts were evaporated to dryness under nitrogen at 40 °C and reconstituted in methanol (50 µL).

Data Analysis

Calibration using deuterated internal standards was calculated using linear regression analysis over a concentration range of 25 to 10,000 pg/mg for all drugs. Peak area ratios of target analytes and their respective deuterated standards were calculated using Mass Hunter software (Agilent). The data were fit to a linear least-squares regression curve with a 1/x weighting and was not forced through the origin.

Selectivity

Drug-free hair specimens were obtained from volunteers and extracted and analyzed according to the described procedures in order to assess interference from extraction or matrix, or potential ion suppression. Ion suppression is not as prevalent using APCI as it is in electrospray mode. In addition, interferences from commonly encountered drugs were added to the drug-free hair specimens and subjected to the same extraction and analysis procedures. The following drugs were analyzed using the described procedures at a concentration of 20,000 pg/mg: morphine, 6-acetylmorphine, codeine, hydrocodone, hydromorphone, oxycodone, oxymorphone, tramadol, desmethyltramadol, fentanyl, gamma-hydroxybutyrate (GHB), tetrahydrocannabinol (THC), 9-carboxy-THC, amphetamine, methamphetamine, methylenedioxymethamphetamine (MDMA), methylenedioxyamphetamine (MDA), methylenedioxyethylamphetamine (MDEA), carisoprodol, methadone, phencyclidine, diazepam, nordiazepam, oxazepam, alprazolam, chlordiazepoxide, bromazepam, temazepam, lorazepam, flurazepam, 7-aminoflunitrazepam, α-hydroxyalprazolam, nitrazepam, triazolam, α-hydroxytriazolam, amitryptiline, nortriptyline, imipramine, protriptyline, doxepin, nordoxepin, trimipramine, secobarbital, pentobarbital, butalbital, and phenobarbital.

Linearity and Sensitivity

The linearity of the assays was established with eight calibration points, excluding the drug-free matrix. The sensitivity of the method was deter-

mined by establishing the limit of quantitation (LOQ), defined as the lowest concentration detectable with a signal-to-noise (S:N) ratio of at least 10 and retention time within 0.2 minutes of the calibration standard. The limit of detection (LOD) was determined from the lowest concentration detectable with an S:N ratio of at least 3.

Precision

Inter- (between day) and intra-day (same day) precision of the assays was determined at the calibration point of 100 pg/mg for all drugs. Intra-day data were obtained from five analyses performed on one day; inter-day data were obtained by analyzing a total of 10 specimens over 5 days (2 samples per day for 5 days; n = 10).

Stability

The stability of the drug extracts at a concentration of 50 pg/mg was determined by allowing the autosampler vials to remain in the liquid chromatographic chamber for 48 hours, after which time they were re-analyzed. The unit was maintained at 4 °C. The responses were compared to those achieved on the first day of analysis.

Application to Authentic Specimens

As part of various ongoing research studies, our laboratory receives hair specimens for research purposes as well as proficiency specimens.

LC/MS Method Details

LC Conditions

Agilent 1200 Series binary pump, degasser, thermostat-controlled wellplate sampler, and thermostatted column compartment.

Column:	Agilent ZORBAX XDB-C18, 4.6 x 50 mm, 1.8 µm (p/n 922975-902)
Column temperature:	40 °C
Mobile phase:	A = 20 mM ammonium acetate (pH 6.4) B = methanol
Flow rate:	0.9 mL/min
Injection vol:	2 µL

Gradient:

Time (min)	%B	Flow (mL/min)	
0.0–1.5	30	0.9	
4.5	55	1	
5	60	1	Stop time: 7 min
7	75	1	Post time: 6 min

Needle wash (75:25 methanol/water): flush port 2 seconds

MS Conditions

Agilent 6410 Triple Quadrupole Mass Spectrometer (QQQ)

Mode:	Positive APCI using the Agilent G1947B ionization source
Vaporizer temperature:	400 °C
Drying gas flow:	5 L/min
Drying gas temperature:	350 °C
Nebulizer:	50 psig
V_{cap}:	4500 V
Corona needle:	4 µA
Resolution (FWHM):	Q1 = 2.5 amu; Q2 = 0.7 amu

Dwell time for all MRM transitions = 50 msec

Two transitions were selected and optimized for each drug using flow injection analysis. One parameter requiring optimization is the fragmentor voltage, which is located between the ion source and the QQQ mass analyzer. This voltage needs to be optimized for maximum transfer of the precursor ions into the first quadrupole of the mass analyzer. For all compounds this value was determined to be 120 V.

Table 1 shows the optimized collision energy voltages for each precursor ion (M + 1) to produce the quantifier and qualifier product ions. Each subsequent analysis required the ratio between the quantitative ion and the qualifier ion to be within ± 20% in order to meet the criterion for a positive confirmation. The ion ratio for each drug was

Table 1. MRM Mode Parameters (Values for qualifiers in parentheses)

Segment	Compound	Transition	Collision Energy (V)
1 (0 min)	Benzoylecgonine	290.3 > 168.3 (105.3)	15 (15)
	D3-Benzoylecgonine	293.3 > 171.4	20
2 (3.2 min)	Not used		
3 (4 min)	Cocaine	304.3 > 182.3 (82.2)	20 (25)
	D3-Cocaine	307.3 > 185.3	20
4 (4.9 min)	Cocaethylene	318.3 > 196.4 (82.2)	25 (25)
	D8-Cocaethylene	326.3 > 204.4	20
	Norcocaine	290.3 > 168.3 (136.3)	15 (25)
	D3-Norcocaine	293.3 > 171.4	15

determined at the concentration level of 100 pg/mg.

Results and Discussion

Method Development

The development of simple LC/MS/MS assays for the detection of COC and its metabolites in hair is reported. While these drugs have been detected in hair, the increasing utility of LC/MS/MS in laboratories makes development of confirmatory procedures necessary and timely. The monitoring of a second qualifying ion is reported for the first time for COC hair analysis, and is necessary for the improved confidence in the identification of the analyte. An example is shown in Figure 2.

Method Validation

The chromatographic procedures developed for COC, BZE, CE, and NC were validated according to accepted protocols. The limit of quantitation for each drug and calibration curve data were determined as described in the Experimental section. Linearity was obtained with an average correlation coefficient for all the drugs of $R^2 > 0.99$ over the concentration range from 25 to 10,000 pg/mg of hair. An example is shown for CE in Figure 3.

Table 2 shows the mean correlation, equation of the slope of the calibration curve, and the qualifying ratio between the transitions monitored. The low intensity of the second transition for BZE (6.7 to 10%) limited the sensitivity of the method for that particular drug; however, the importance of having a qualifying transition was considered to be of greater importance in forensic identification than sensitivity.

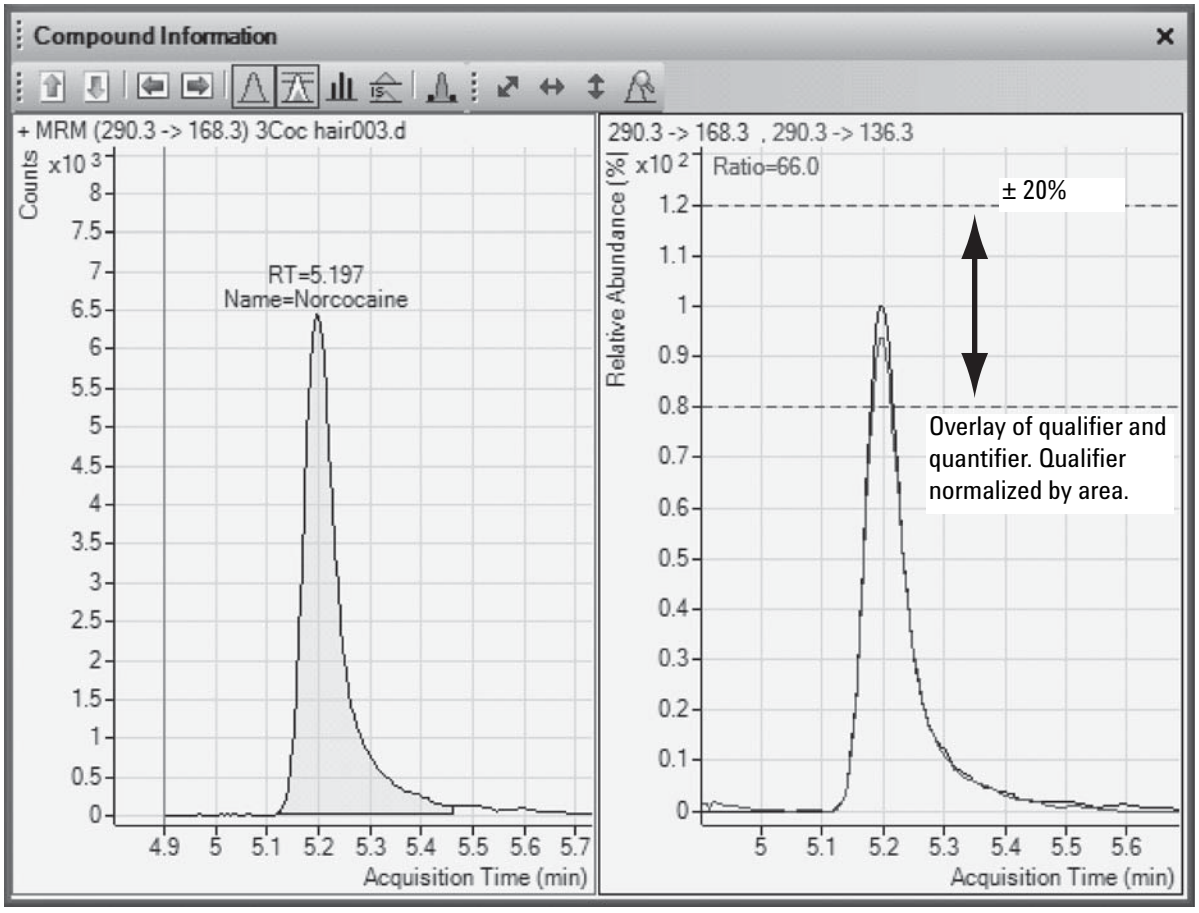

Figure 2. Ion ratio confirmation for norcocaine at 100 pg/mg level.

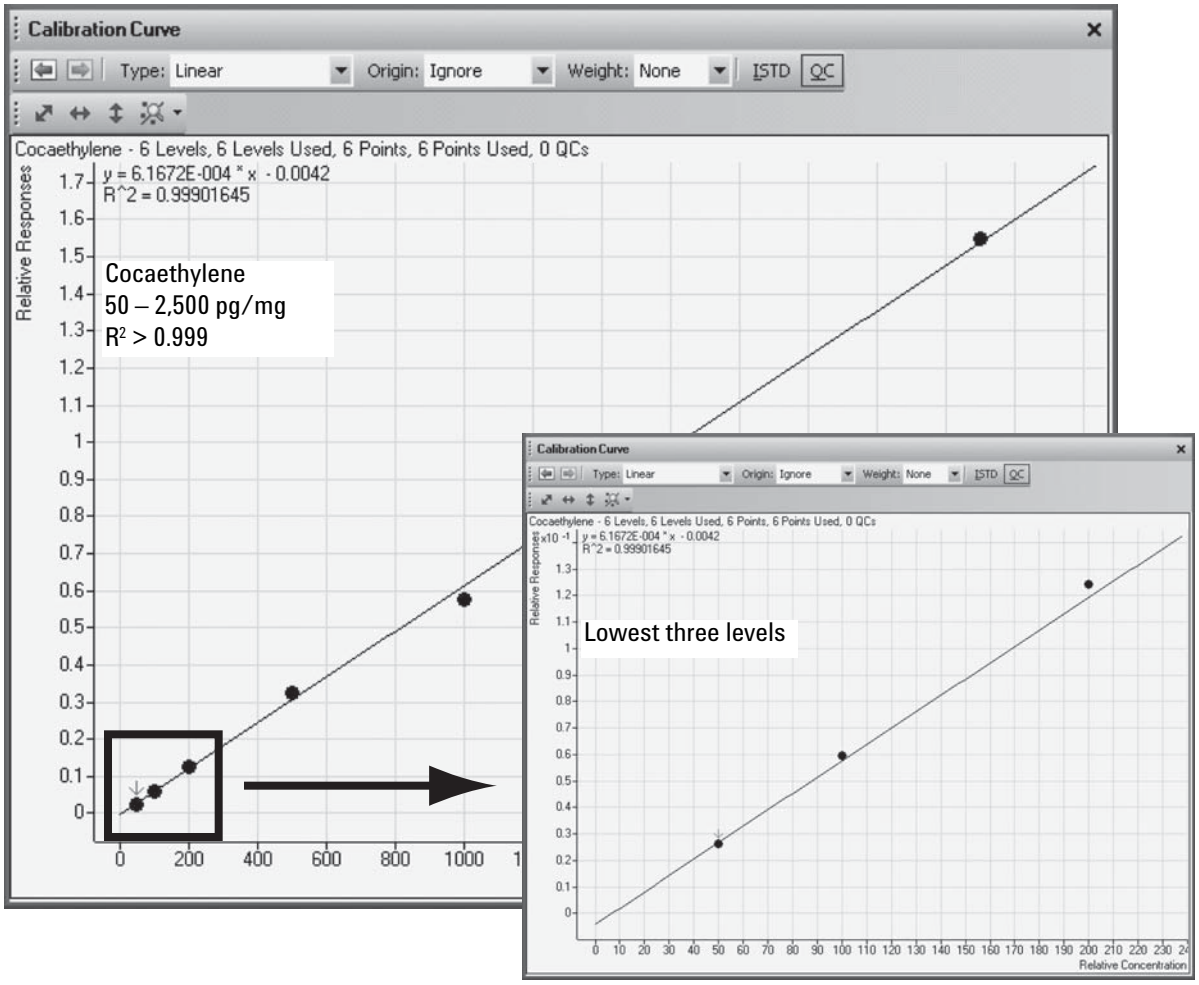

Figure 3. Linearity of cocaethylene with lowest levels detailed to show excellent accuracy.

Table 2. Mean Correlation, Equation of the Slope of the Calibration Curve and the Qualifying Ratio Between the Transitions Monitored

Drug	Mean correlation (n = 3)	Equation for calibration curve	Allowable range of intensity for qualifying ion
Benzoylecgonine	0.9989	y = 0.00116x	6.7–10%
Cocaine	0.9995	y = 0.00106x	37.8–56.8%
Cocaethylene	0.9987	y = 0.00061x	49.3–74%
Norcocaine	0.9992	y = 0.00096x	52.8–79.2%

Hair specimens collected from drug-free individuals showed no interference with any of the assays, which was not unexpected, since it is unlikely these drugs are similar to endogenous substances in hair. For exogenous interferences, commonly encountered drugs of abuse were studied as described in the Experimental section. No chromatographic interference was observed in the channels of these transitions.

An example of an extracted hair specimen at a concentration of 50 pg/mg is shown in Figure 4. The inter-day and intra-day precision of the assays was determined using replicate analyses as described. For BZE, COC, CE, and NC, the inter-day precision was 9.2%, 4.8%, 15.7%, and 12.6%, respectively (n = 10). For same-day precision (n = 5), the values were 8.1%, 1.3%, 0.8 %, and 0.4%, respectively. Finally, the stability of the drugs

in the collection system and the stability of the extracts were assessed. The extracts were stable for at least 2 days when kept in the instrument rack inside the autosampler, which was maintained at 7 °C. There was less than a 5% difference in the quantitation of the extracts after 48 hours.

Authentic Specimens

The procedures were applied to proficiency specimens received into the laboratory. The performance was excellent, with all quantitation being within 10% of the group mean identified by the program administrators.

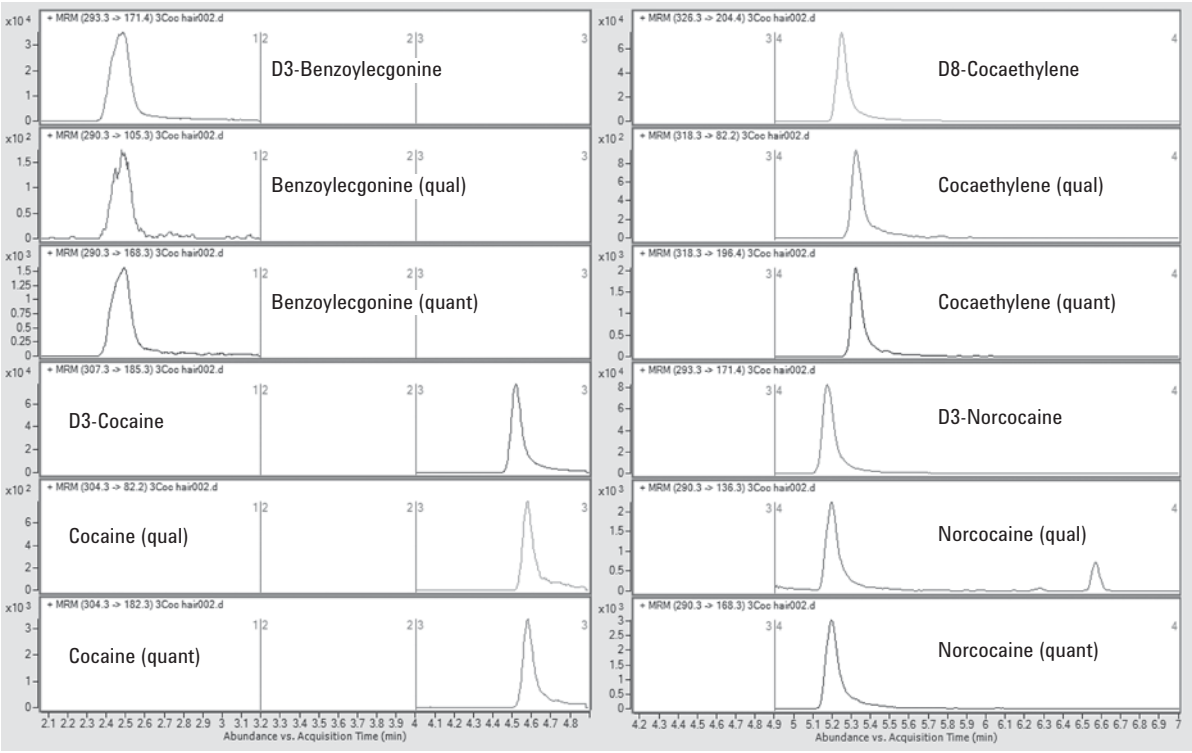

Figure 4. Chromatographic profile of all compounds analyzed in hair at the 50 pg/mg level.

Conclusions

The determination of COC, BZE, CE, and NC in hair is described. The LC/MS/MS procedure is reproducible, robust, and precise. The assay includes the monitoring of a qualifying transition and calculation of a ratio, required to be within 20% of that of a known calibration standard in order for definitive identification to be made. The method is easily incorporated into routine laboratory testing.

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Printed in the USA
September 27, 2007
5989-7212EN

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