

High-Throughput GC/MS Confirmation and Quantitation of Benzoyllecgonine in Urine Using the DSQ II

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Abstract

Commonly abused for its strong effects as a central nervous system stimulant, cocaine is a drug whose use is closely monitored in the United States and elsewhere. It is extracted and isolated from the South American shrub, *Erythroxylon coca*. Besides its use by abusers, cocaine has been utilized by the medical industry as a local anesthetic through topical application of its hydrochloride salt. When abused, it is usually taken either as the hydrochloride through injection or nasal inhalation or by smoking the free base.¹

Workplace drug testing laboratories typically will look in urine for indications of its use. This is generally done by monitoring a primary metabolite, benzoylecgonine (BE), since in urine this compound is found in much higher concentration than cocaine after its use. The methodology described below details the confirmation and quantitation of benzoylecgonine (Figure 1) in urine using the DSQ™ II GC/MS system.

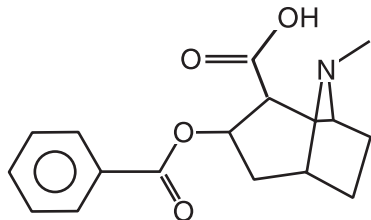


Figure 1: Chemical structure of benzoylecgonine

For this assay, a 2 mL urine sample size was used, with benzoylecgonine-D3 as the deuterated internal standard. Samples were extracted using solid phase extraction. After extraction, the samples were derivatized with hexafluoroisopropanol (HFIP), using pentafluoropropionic acid (PFPA) as a catalyst. The final reaction products were analyzed using a DSQ II single stage quadrupole GC/MS system. A calibrator at 150 ng/mL was used for single point calibration. The resulting method demonstrated excellent precision, no interference for a number of tested compounds and provided linearity from 15 to 12,500 ng/mL, with a limit of detection and limit of quantitation of 15 ng/mL.

Introduction

Cocaine in its parent state has a strong stimulating effect on humans. When cocaine enters the body, it is deactivated by the hydrolysis of one or more of its methyl esters to form, among other things, benzoylecgonine. The presence of benzoylecgonine in a urine sample identifies the donor as one who has been exposed to some form of cocaine, either through self administration or via indirect contact.¹ Because benzoylecgonine contains a carboxyl functional group that does not lend itself well to gas chromatography, samples for BE confirmation are typically derivatized, which allows laboratories to realize increased productivity and take full advantage of the speed and ease of use that is afforded by GC/MS.

The DSQ II, a single stage quadrupole mass spectrometer with a curved prefilter that minimizes background noise derived from excited neutrals, was used for this analysis. Coupled to a TRACE GC Ultra™ gas chromatograph and an AS3000 autosampler, this GC/MS system represents the standard for confirmatory analyses of drug use. ToxLab™ 2.0 Intelligent Sequencing Software provided automated sample analysis and quantitation, and the method was fully validated, including assessments of precision, interference, and linearity. This method describes the GC/MS confirmation and quantitation of benzoylecgonine in urine, and it does not include other matrices or other cocaine metabolites, nor does it encompass the analysis of cocaine. The method here utilizes HFIP/PFPA for derivatization, which caps the labile hydrogen of the carboxylic acid group with HFIP.

Methods

To provide a comprehensive view of method development and validation, methods for sample preparation, acquisition, and analysis are described in detail below. Sample preparation plays a critical role in method validation since many certifying bodies recommend or require method validation performed in matrix. In this case, solid phase extraction is used due to its ease of use and the cleanliness of the resultant extracts.²

Key Words

- DSQ II GC/MS
- ToxLab 2.0 Software
- Benzoylecgonine
- Cocaine
- Urine Drug Testing

Sample Preparation

Known negative urine was collected and used for sample preparation. A sample size of 2 mL was selected. Calibrators, quality controls, and linearity samples were spiked with appropriate amounts of benzoylecgonine (Cerilliant, Round Rock, TX). Single point calibration at 150 ng/mL was used for calculation of all quantitative amounts. A commercial control (Medical Analysis Systems, Level G3, Freemont, CA) calibrated to represent 125% of 150 ng/mL (187.5 ng/mL) was used as the positive control for the batch, and the 40% control (60 ng/mL) was prepared from BE source material from an alternate source (Alltech Associates, Deerfield, IL). All batches contained an unextracted standard, the calibrator at 150 ng/mL, a negative control, a 40% control and a 125% control. BE-D3 (Cerilliant) was used as the deuterated internal standard, and was added to each sample at a final concentration of 150 ng/mL. An unextracted standard was prepared by adding 100 μ L of 3 μ g/mL BE standard solution and 100 μ L of 3 μ g/mL BE-D3 internal standard solution to a labeled tube, yielding the equivalent of a 150 ng/mL sample. The purpose of the unextracted standard is to demonstrate recovery, to prep the GC/MS system, and to demonstrate ion ratios. The unextracted standard is not subjected to the extraction steps but instead proceeds directly to the dry-down step, at which point it rejoins the rest of the samples for derivatization and analysis.

Prior to extraction, samples were brought to a pH of 6 ± 0.5 by adding 2 mL of 0.1 M sodium phosphate buffer, pH 6. Each sample was then extracted by solid phase extraction on Hypersep™ Verify™ CX columns (Thermo Scientific, PN 60108-742). The extraction columns were conditioned with sequential rinses of the following: 3 mL methanol, 3 mL deionized (DI) water, and 1 mL 0.1 M phosphate buffer pH 6. Between each conditioning step, the columns were not allowed to dry. The pH-adjusted samples were then loaded onto the column and extracted under low vacuum (≤ 3 in. Hg). After the samples were loaded, the columns were washed sequentially with 2 mL of DI water, 2 mL of 0.1 M HCl and 3 mL of methanol. The columns were then dried under high vacuum for five minutes. Finally, sample

eluent were collected in clean tubes under low vacuum (≤ 1 in. Hg) with 3 mL of elution solvent (methylene chloride-isopropanol-ammonium hydroxide, 78:20:2 v:v:v).²

The extracts were evaporated to dryness at 40 °C under nitrogen. Caution was taken to prevent excessive drying of the extracts. Next, the dried samples were derivatized by adding 50 μ L of PFPA and 25 μ L HFIP and heating at 90 °C for 10 minutes. The derivatized samples were evaporated at 40 °C under nitrogen. For analysis, 50 μ L of ethyl acetate were added to the derivatized extracts, and the resulting samples were transferred to autosampler vials with glass inserts and loaded onto the AS3000 autosampler for GC/MS analysis. Table 1 summarizes sample prep, extraction, and derivatization steps.

Instrumental Analysis

The DSQ II mass spectrometer used for this analysis was configured with a 250 L/s turbomolecular pump, and the TRACE GC Ultra was equipped with a standard split/splitless injector. A 5 mm i.d. deactivated glass liner was used in the injector (Thermo Scientific, PN 45350033), and glass wool was used in the liner. The split/splitless injector temperature was set to 275 °C. A 1 μ L injection volume was programmed on the AS3000 autosampler, and a 20:1 split injection was used. The analytical column was a TRACE™ TR-DoA5 15 m x 0.25 μ m i.d. x 0.25 μ m film thickness (Thermo Scientific, PN 260F130P), which was installed 64 mm into the injection port (Figure 2). A plug of glass wool was placed approximately 40 mm from the top of the liner.

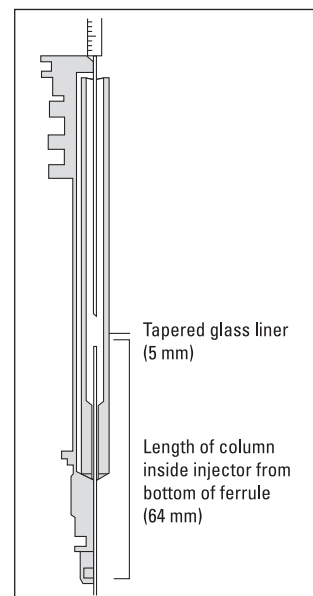


Figure 2: Column installation in GC split/splitless injection port (not to scale)

Sample Preparation

1. Label 13 x 100 mm screw top culture tubes
2. Add 2 mL of blank urine, QC or donor specimen
3. Add 100 μ L of working BE-D3 internal standard to each tube
4. Spike calibrator and low QC with BE
5. Add 2 mL of 0.1 M pH 6 phosphate buffer
6. Vortex gently
7. Prepare vacuum manifold for sample extraction

Extraction

1. Condition SPE columns sequentially with
 - a. 3 mL methanol
 - b. 3 mL DI water
 - c. 1 mL 0.1 M pH 6 phosphate buffer
2. Apply samples at low vacuum
3. Rinse SPE columns sequentially with
 - a. 2 mL DI water
 - b. 2 mL 0.1 M HCl
 - c. 3 mL methanol
4. Dry columns at high vacuum for 5 minutes
5. Elute BE with 3 mL 78:20:2 methylene chloride: isopropanol: ammonium hydroxide; collect in labeled culture tubes

Concentration and Derivatization

1. Evaporate samples at ≤ 40 °C under N_2 stream until dry
2. Add 50 μ L PFPA and 25 μ L HFIP
3. Cap culture tubes, vortex and heat at 90 °C for 10 minutes
4. Remove from heat, let cool and blow down excess PFPA/HFIP
5. Add 50 μ L of ethyl acetate and vortex
6. Transfer resulting extracts to autosampler vials with inserts for GC/MS analysis

Table 1: Sample Prep, Extraction and Derivatization Summary

The programmed carrier gas flow started with an initial flow rate of 2.5 mL/min of helium. At 1.30 minutes, the flow was ramped to 12.5 mL/min to get the heavy matrix compounds through the column as quickly as possible. The initial temperature on the TRACE GC Ultra was set to 175 °C. The high temperature at the beginning of the analytical run allowed the BE to elute from the column as quickly as possible. Upon injection, the GC temperature was immediately ramped at 60 °C/min to a final temperature of 320 °C and held for 0.6 min, for a total run time of 3.02 minutes, and a BE retention time of 1.13 minutes.

The DSQ II source temperature was set to 300 °C, and the mass spectrometer was tuned using default *AutoTune* parameters. These tune settings were used for acquisition, with a detector gain of 1×10^5 . For initial mass spectrometer method development, high concentrations of derivatized BE and BE-D3 were injected and analyzed in electron impact (EI) full scan to determine masses for EI selected ion monitoring (SIM). The set of SIM masses and dwell times used to detect BE and its deuterated internal standard are shown in Table 2. Mass 318 was used as the quantitation mass for BE, and mass 321 was the quantitation mass for internal standard, BE-D3. The narrow SIM width used enhances sensitivity and builds on the mass stability and resolution of the DSQ II, while a short dwell time provides quantitative precision across the narrow GC peak that results from the use of fast GC. Table 2 summarizes instrument parameters for the validated method.

Sample Processing and Result Derivation

For sample acquisition, peak detection and quantitation, ToxLab 2.0 Intelligent Sequencing Software was utilized. By incorporating all vital components of analyses into a unified workflow-oriented application, ToxLab 2.0 provides an integrated solution to benzoylecgonine GC/MS confirmation.

DSQ II	
Source Temp (°C):	300
Acquisition Time (min):	1.35
Detector Gain:	1×10^5
Start Time (min):	1
BE Mass (<i>m/z</i>):	318.0 272.0 334.0
BE-D3 Mass (<i>m/z</i>):	321.0 442.0
Width (amu):	0.5
Dwell Time (ms):	10

TRACE GC Ultra	
Oven Method	
Initial Temp (°C):	175
Initial Time (min):	0.0
Rate (°C /min):	60
Final Temperature (°C):	320
Final Hold Time (min):	0.6
SSL Method	
Temperature (°C):	275
Mode:	Split
Split Ratio:	20:1
Split Flow:	50
Constant Septum Purge:	on
Carrier Method	
Initial Value (mL/min):	2.5
Initial Time:	1.3
Rate #1 (mL/min ²):	999.9
Hold Time #1 (min):	5
Final value (mL/min):	12.5
Gas Saver:	on
Gas Saver Flow (mL/min):	200
Gas Saver Time (min):	0.5
Vacuum Compensation:	on
Transferline Temp (°C):	280

AS3000	
Sample Volume (µL):	1
Plunger Strokes:	5
Viscous Sample:	Yes
Sampling Depth in Vial:	Bottom
Injection Depth:	Standard
Pre-Inj Dwell Time (sec):	0
Post-Inj Dwell Time (sec):	0
Sample Rinses:	0
Pre-Injection Solvent Rinses:	0
Post-Inj Solvent Rinses	
Solvent A (50:50 EtOAc:MeCl ₂):	3
Solvent B (50:50 EtOAc:MeCl ₂):	3

Table 2: Instrument method summary for the SIM analysis of benzoylecgonine on the DSQ II

To make use of ToxLab 2.0 for method validation, an instrument method was created for the mass spectrometer, autosampler, and GC. A processing method for component identification and quantitation was developed. In ToxLab 2.0, these methods were integrated into a single master method, which also allows the user to establish criteria specific to the method. Batch creation was performed through the *Batch Wizard* function of ToxLab 2.0, which greatly simplified and streamlined sample entry, particularly for the longer validation batches (Figure 3). This highlights the applicability of this software to routine analysis of toxicological samples.³

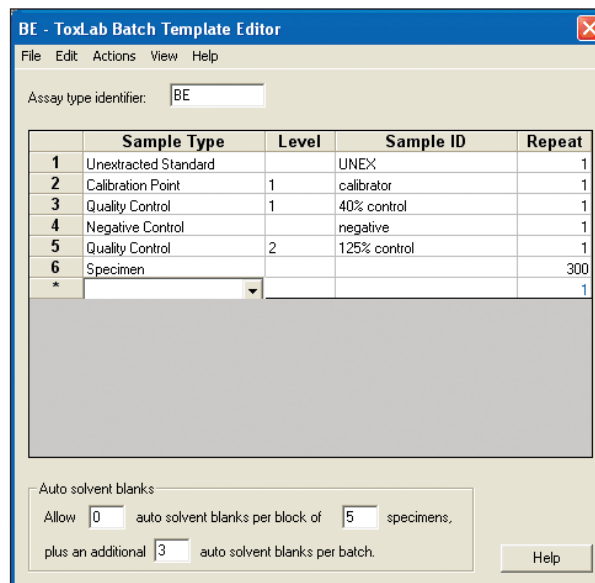


Figure 3: ToxLab 2.0 Batch Template Editor, showing framework for benzoylecgonine batches

Concentration calculations were based on a single point calibrator at 150 ng/mL, using BE-D3 as the internal standard. Linear calibration including the origin created the calibration curve, and calculated amounts were based on this curve. All validation batches had to conform to quality control (QC) criteria, including quantitative and qualitative bounds checking.

Quantitative criteria for the batch included acceptable quantitation ranges for all samples in each batch. All calculated amounts for QC samples and study samples had to fall within $\pm 20\%$ of the expected concentration in order to accept the sample. Failure of a QC sample within a batch would mean the entire batch would need to be repeated. In addition to this quantitative window, negative controls were evaluated based on two additional criteria. One means of assessing a negative control is a quantitative value for BE less than the method limit of detection (LOD), which in this case is 15 ng/mL. An alternate criterion for negative controls is that the calculated amount must be less than a pre-determined percentage of the method cutoff. For this method, a level of 5% of the cutoff (7.5 ng/mL) was used as a second criterion, and all negative controls were evaluated for compliance to both criteria.

Qualitative criteria included ion ratio and retention time target ranges based on the calibrator, along with peak shape considerations. These criteria were applied to all sample types. Ion ratio ranges for the batch were developed based on the appropriate ratios from the 150 ng/mL calibrator. Ratios were defined as follows:

$$\text{ion ratio} = \frac{\text{area of qual ion}}{\text{area of quant ion}} \times 100\%$$

Ratios were calculated for BE-D3 (442:321) and BE (272:318 and 334:318), and for each ratio, an acceptable range of $\pm 20\%$ was established. Similarly, the target retention time for BE and BE-D3 was set using a $\pm 2\%$ retention time window based on the calibrator retention time. Peak symmetry requirements required the peaks to be $>90\%$ symmetrical at 50% peak height.

Each validation batch was reviewed for compliance with these criteria, and for a study batch to be accepted, it had to comply with all of these QC criteria.

Results

The analysis of benzoylecgonine in urine using the DSQ II GC/MS system was thoroughly validated through determination of linear range, carryover, precision, and specificity. Four separate batches were prepared and analyzed: one for linearity/carryover, one for specificity, and two for precision. Each batch included the appropriate quality controls and calibration standards, along with validation samples prepared according to Table 3. Batch acceptability was determined by applying the QC standards described above. Carryover was assessed during the course of the linearity study. Precision analyses were performed on two separate batches analyzed on two separate days, while specificity assessed potential interference from a number of compounds. The DSQ II demonstrated excellent intra- and inter-day precision, linearity from 15 to 12,500 ng/mL, with carryover below the QC limits following 12,500 ng/mL, and no interference was seen for this assay for the compounds tested. With 5.9 minute inject-to-inject times, the method also provides a productive means of performing this confirmation.

Linearity	Precision	Interference
1. Unextracted (150 ng/mL)	Batch 1	1. Unextracted (150 ng/mL)
2. Calibrator (150 ng/mL)	1. Unextracted (150 ng/mL)	2. Calibrator (150 ng/mL)
3. 40% Control (Alltech)	2. Calibrator (150 ng/mL)	3. 40% Control (Alltech)
4. Negative	3. 40% Control (Alltech)	4. Negative
5. 125% Control (MAS)	4. Negative	5. 125% Control (MAS)
6. 15 ng/mL x 7	5. 125% Control (MAS)	6. Negative w/ Interference #1
7. 30 ng/mL x 7	6. 60 ng/mL x 7	7. 60 ng/mL w/ Interference #1
8. 60 ng/mL x 7	7. 150 ng/mL x 7	8. 187.5 ng/mL w/ Interference #1
9. 150 ng/mL x 7	8. 187.5 ng/mL x 7	9. Negative w/ Interference #2
10. 250 ng/mL x 7	Batch 2	10. 60 ng/mL w/ Interference #2
11. 500 ng/mL x 7	1. Unextracted (150 ng/mL)	11. 187.5 ng/mL w/ Interference #2
12. 1,250 ng/mL x 7	2. Calibrator (150 ng/mL)	12. Repeat for Remaining Interferents
13. 2,500 ng/mL x 7	3. 40% Control (Alltech)	
14. 5,000 ng/mL x 7	4. Negative	
15. 12,500 ng/mL x 7	5. 125% Control (MAS)	
	6. 60 ng/mL x 7	
	7. 150 ng/mL x 7	
	8. 187.5 ng/mL x 7	

Table 3: Validation study sample preparation guide for BE confirmation in urine

Linear Range Determination

The determination of assay linearity was performed at concentrations across a broad dynamic range. The linearity batch, as with every validation batch, included an unextracted standard, a negative control (blank urine and internal standard), the 150 ng/mL calibrator, a 40% control sample (60 ng/mL) and a 125% commercial control sample (187.5 ng/mL). To evaluate method linearity, samples at 15, 30, 60, 150, 250, 500, 1250, 2500, 5000 and 12,500 ng/mL were prepared and extracted, along with the calibrator and controls. These samples were then injected 7 times each, and the resulting 70 data points were quantified based on the 150 ng/mL calibrator. All 70 quantitative values were within $\pm 20\%$ of their target concentrations, and a regression analysis comparing the average quantitative value for each level to its expected value was found to have a correlation coefficient of 0.9994 (Figure 4). At the lowest level, 15 ng/mL, the coefficient of variation (CV) of the calculated amount was 1.1%, with an average concentration of 15.4 ng/mL. Chromatography for the quantitation ion and all qualifiers was exceptional, as shown in Figure 5.

In addition to measuring quantitative performance, the ratios of the qualifier ions to the quantitation ion for both BE and BE-D3 were also evaluated across the concentration range. For BE, m/z 318 served as the quantitation mass, while m/z 272 and 334 were used for confirmation. m/z 321 was used as the quantitation mass for the internal standard, with m/z 442 used as the BE-D3 confirmatory ion. The acceptable ion ratio ranges were calculated based on the appropriate ratios from the 150 ng/mL calibrator, and a relative range of 20% was used as evaluation criteria for the 70 linearity injections. For each of these injections, the ion ratios were calculated, and all were found to be within the acceptable range, indicating excellent linearity of ion ratios across the concentration range.

An additional component of the linearity study included a determination of the carryover limit for the method. To do so, a negative control was injected following each set of linearity samples. These negatives were evaluated for acceptability according to the batch criteria described above. Under these constraints, there was no carryover failing the QC criteria even following the 7 injections of the 12,500 ng/mL level. The use of a gas-tight syringe coupled with syringe rinse steps ensures minimal carryover. [Note: A small response is seen on all negatives due to contribution from minor ions of the deuterated internal standard.]

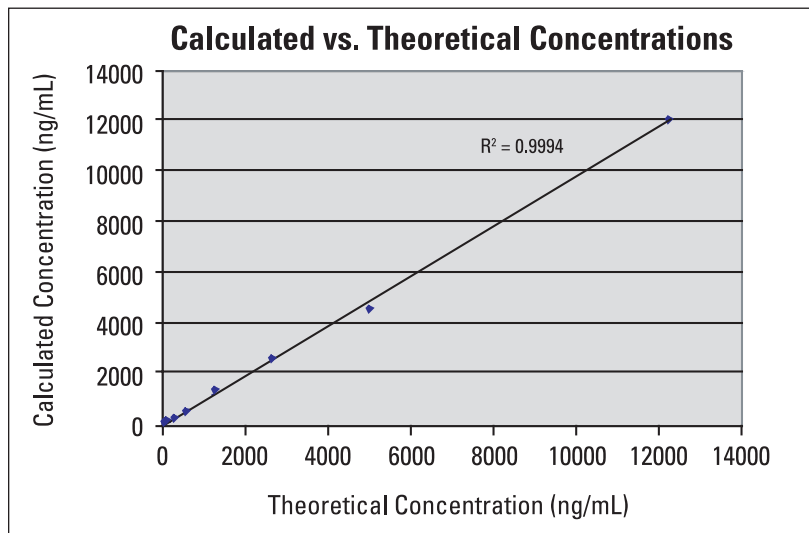


Figure 4: Linearity study results, comparing average concentrations for replicates at 10 different levels to the nominal amounts at each level. The regression analysis for this study gave a correlation coefficient of 0.9994 from 15 ng/mL to 12,500 ng/mL.

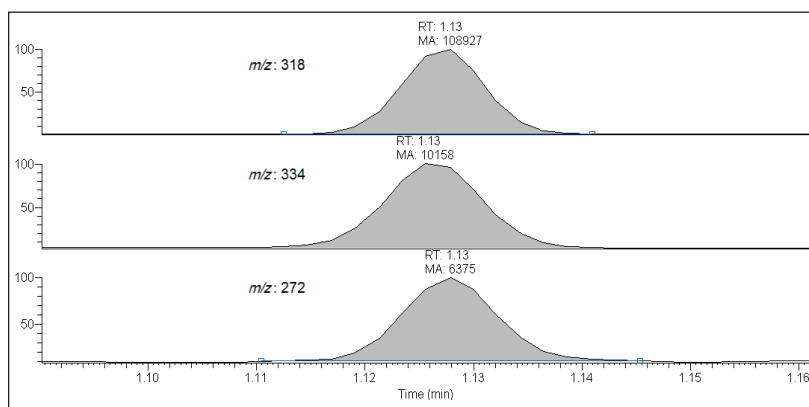


Figure 5: m/z 318, 334, and 272 from the 15 ng/mL level, showing good chromatography and signal intensity at the limit of detection for this method

Finally, for the batch to be considered acceptable, the quality control for the batch had to meet QC standards described above. For the 40% control, the calculated value was 61.5 ng/mL, a 3% deviation from the target and well within the $\pm 20\%$ quantitation range, and the ion ratios were also within the $\pm 20\%$ target range. The 125% control was calculated to be 182 ng/mL, also a 3% deviation from nominal and within $\pm 20\%$, and the ion ratios met their criteria. The negative control was also acceptable. As such linearity batch was accepted. Table 4 includes a summary of the linearity/carryover study for BE on the DSQ II.

Expected Concentration (ng/mL)	Average Calculated Concentration (ng/mL)
15	15.4
Negative	1.3
30	30.7
Negative	1.1
60	58.7
Negative	1.2
150	150
Negative	1.3
250	253
Negative	1.3
500	495
Negative	1.2
1,250	1,270
Negative	1.3
2,500	2,470
Negative	1.8
5,000	4,560
Negative	1.5
12,500	12,100
Negative	2.8

Table 4: Results of linearity/carryover study. Calculated concentrations representing points on the linearity curve were obtained by averaging seven injections made at that concentration.

Intra- and Inter-day Precision

Instrument precision and method precision were measured by extracting two separate precision batches and running these batches on two different days. The precision study was designed to indicate precision at the 40% level, at the cutoff of 150 ng/mL and at the 125% level. Coefficients of variation (CV) were calculated for the average concentrations at each level, and these CVs were required to be less than 10% for each concentration. As with the linearity batch, the precision batches had to comply with the QC criteria, and all controls were acceptable. To gauge inter-day precision, the CVs from the combined data of the two precision batches had to be less than 10%.

The method described above provides excellent quantitative precision, with intra-batch CVs all less than 1%, and inter-batch CVs all less than 3%. Table 5 includes a summary of the precision results for BE on the DSQ II.

Specificity

To determine assay specificity, an interference study was also performed. A number of compounds with potential to interfere with the immunoassay screening test for BE were included in this test, as were a range of other drugs. Table 6 describes the drugs and their respective concentrations. Cocaine and other cocaine metabolites were assessed individually. The other drugs were analyzed together. For each interference test, the potential interferent was spiked into a blank urine sample, a 60 ng/mL sample and a 187.5 ng/mL sample at the concentration specified. All negatives met the negative control criteria for BE, and each 40% and 125% control quantified within 20% of the target concentration, showing that none of the potential interferents tested affected quantitation. Also, all ion ratios were checked against the ion ratios of the calibrator and each were within 20% of the calibrator ion ratios, showing no interference with the confirming ions. Retention times also fell within the specified window of $\pm 2\%$ of the calibrator retention time. The interference batch also complied with all applicable QC criteria, and the results of the specificity batch were accepted as demonstrating the assay to be free of interference from the tested compounds.

Concentration	CV for Batch 1	CV for Batch 2	Inter-batch CV
60 ng/mL	0.5%	0.6%	0.7%
150 ng/mL	0.8%	0.4%	1.1%
187.5 ng/mL	0.5%	0.8%	2.3%

Table 5: Results of precision study showing intra-day coefficients of variations less than 1% and inter-day coefficients of variation for inter-batch calculated amounts less than 3%

Drug	Concentration (ng/mL)
Cocaine	1000
Ecgonine methyl ester	1000
Ecgonine	1000
Norcaine	1000
Ethosuximide	1000
α -Methyl- α -propylsuccinimide	1000
Metharbital	1000
Barbital	1000
Methsuximide	1000
Phensuximide	1000
Normethsuximide	1000
Mephentoin	1000
Ethotoin	1000
Mephobarbital	1000
PEMA	1000
Phenobarbital	1000
Methyl PEMA	1000
10,11-Dihydrocarbamazepine	1000
Primidone	1000
Phenytoin	1000
Carbamazepine	1000
4-Methylprimidone	1000
Caffeine	667
Methadone	500
Codeine	833
6-Monoacetylmorphine	1250
Diacetylmorphine	1250

Table 6: List of compounds tested for potential interference, along with concentrations tested

Conclusion

The analysis of benzoylecgonine on the DSQ II was completed with a BE retention time of less than two minutes. The validated method shown is very sensitive, with a wide dynamic range, ranging from 15 to 12,500 ng/mL. All samples tested in this range gave calculated amounts that were within 20% of the nominal values, based on a one-point calibration curve at 150 ng/mL. Across this range, all samples also gave ion ratios which were within 20% of the ion ratios of the calibrator. A series of replicate injections at the reported LOD/LOQ of 15 ng/mL gave a coefficient of variation of 1.1% and an average calculated value of 15.4 ng/mL, demonstrating remarkable sensitivity even when using a split injection technique. Method precision and specificity were also excellent, with intra-day coefficients of variation all less than 1% at three different concentrations. Because all method development and validation were performed in extracted urine matrix, the results demonstrate that the DSQ II is able to handle matrix contamination if a sufficient amount of sample preparation is done. These results also accurately reflect method development and validation as they would be performed within a working laboratory.

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