

Determination Of Opioids, Cocaine, and Cocaine Metabolites by Liquid Chromatography Mass Spectrometry Using ZORBAX Eclipse Plus C18 Columns

Application

Forensics

Authors

Patrick Friel B.S. and Ann Marie Gordon M.S.
Washington State Toxicology Laboratory
Forensic Laboratory Services Bureau
Washington State Patrol
2203 Airport Way South, Suite 360
Seattle, WA 98134
USA

Abstract

An improved method for the analysis of opioids, cocaine and cocaine metabolites from blood using solid phase extraction followed by LC/MS is described. An Eclipse Plus C18 column is used to separate the drugs and metabolites. The combination of excellent peak shape and resolution afforded by this column together with the sensitivity and selectivity afforded by the LC/MS allow a simple extraction without derivitization to be used to separate and quantify these drugs and metabolites in a single analysis.

Introduction

Over 20% of the blood specimens from cases submitted to the Washington State Toxicology Laboratory are positive for opiates, cocaine metabolites,

or both by immunoassay screens. Death investigations and drug-impaired driving arrests account for the majority of these cases, with a smaller number of sexual assault cases also encountered. Drug concentrations vary widely from case to case, and the analytes appear in many different combinations. The ideal confirmatory analysis should allow determination of all available opioids, cocaine, and cocaine metabolites in a single blood specimen, with high sensitivity and a wide linear dynamic range. Until recently, gas chromatography-mass spectrometry was the industry standard for these confirmations; however, sample derivatization or even dual derivatization is required[1].

At the Washington State Toxicology Laboratory, we have employed liquid chromatography/mass spectrometry (LC/MS) with the Agilent MSD SL and the new ZORBAX Eclipse Plus C18 columns for combined analysis of opioids, cocaine, and cocaine metabolites for several thousand cases. This approach has a number of advantages over our previous GC/MS method, including simpler sample preparation, improved sensitivity, and the ability to detect a broader range of opioids in a single analysis.



Experimental

Methods

Extract

Condition Clean Screen extraction column (United Chemical Technologies, CSDAU206)

- 1 × 3 mL Methanol
- 1 × 3 mL DI Water
- 1 × 3 mL 0.1 M KH₂PO₄

Prepare Blood Sample

- (Standards: add working standard and dry down first)
- 50 µL Internal Standard (ethyl morphine 2 µg/mL)
- 1 mL blood
- 3 mL 0.1 M KH₂PO₄
- Vortex mix and centrifuge 2,500 rpm 15 min

Apply diluted, centrifuged blood to conditioned column at 1 to 2 mL/min

Wash Column

- 1 × 3 mL DI water
- 1 × 3 mL 0.1 N HCl
- 1 × 3 mL methanol
- Dry 10 min at maximum vacuum

Elute

- 1 × 3 mL CH₂Cl₂/isopropanol/NH₄OH (72/26/2)
(Prepare fresh daily)

Evaporate @ 50° (~ 20 min) and reconstitute in 100 µL 1% acetic acid.

Chromatographic and Instrument Conditions

Instrument:	Agilent 1100 LC/MSD SL
Column:	ZORBAX Eclipse Plus C18, 4.6 mm × 150 mm, 5 micron (Agilent PN 959993-902)
Mobile Phase:	A: 1% acetic acid B: acetonitrile Start: 3% B At 16.5 min 40% B At 17 min 40% B At 20 min 3% B At 32 min 3% B
Flow rate:	1 mL/min
Column temp.:	60 °C
Injection vol.:	2.5 µL
Needle rinse:	1% acetic acid

MS Conditions

Source:	Electrospray
Ionization mode:	Positive
Vcap:	3,000 V
Nebulizer:	40 psig
Drying gas flow:	13 L/min (nitrogen)
Drying gas temp.:	350 °C
Mass ranges:	SIM, 3 groups Group 1 (1.0 to 4.8 min) 209, 227, 284, 286, 287, 302, 462 amu Group 2 (4.8 to 8.1 min) 181, 241, 257, 268, 298, 300, 314, 316, 328 amu Group 3 (8.1 to 17 min) 168, 196, 272, 290, 291, 304, 318 amu
Fragmentor:	Groups 1 and 2: 260 V; Group 3: 220 V

Results and Discussion

Table 2 gives the retention times and ions used for the compounds in this method. Chemical structures are available in Agilent Application Note 988-4805EN[2]. Raw data files were transferred from the LC/MSD computer to a computer running the Agilent MSD Chemstation for data analysis. (Agilent LC/MS data files are fully compatible with the MSD Chemstation.) For each analyte, one of the target masses represents the pseudomolecular ion formed by proton addition (M+H). Relatively high fragmentor voltages were used in order to produce sufficient qualifier ion abundances by collision-induced dissociation. At least two masses were monitored for each compound, and the acceptable limits for ion ratios were set at ± 25%[3]. In cases where two ions were monitored for a compound, an isotopic mass (M+2) can be used as a third ion, but is not as informative as a qualifier ion representing a known fragment of the target molecule. Under these conditions, sodium adduct formation was not consistent enough to allow M+H+22 ions to be used as qualifier ions. A representative chromatogram of an extract from a control blood specimen is shown in Figure 1.

Table 3 gives the limits of detection, quantitation, and linearity for the method, along with quality control data collected over a six-month period. The laboratory policy is to set acceptance ranges for blood drug controls at ± 20% of the mean value determined in-house. Calibration curve coefficients of determination (r²) were ≥ 0.990 for all of the routinely measured analytes. Recovery of all ana-

Table 2. Compounds Analyzed (pseudomolecular ions in bold type)

Compound	Retention time (min)	Ions monitored
Ethyl morphine (I.S.)	7.47	314 , 257
Morphine	2.96	286 , 227, 209
Hydromorphone	3.83	286 , 227
Codeine	5.55	300 , 241, 181
Oxycodone	6.18	316 , 298, 241
6-acetyl morphine	6.4	328 , 268
Hydrocodone	6.61	300 , 241
Benzoylcegonine	8.62	290 , 168
Cocaine	10.3	304 , 272
Cocaethylene	11.94	318 , 196
Research Compounds		
Morphine-3-glucuronide	1.93	462 , 286
Morphine-6-glucuronide	2.8	462 , 286
Oxymorphone	3.34	302 , 284, 227

lytes except for morphine-3- and morphine-6-glucuronide was > 90%. Recovery of morphine glucuronides was poor (~1%). Carryover from previous injections was noted when extracts were injected without using the needle wash option, but with the needle wash incorporated into the

method, carryover was eliminated at concentrations up to 10,000 ng/mL or higher.

Our methodology is based on that described by Pichini et al[4]. When we attempted to add additional opioids to their procedure, without further modification, severely asymmetric peak shapes were encountered for oxycodone, hydromorphone and hydrocodone. This problem, which has been described previously in the literature, is believed to be due to the formation of multiple adducts with mobile phase constituents[5]. Use of the high-performance Eclipse Plus columns, at a relatively high temperature (60 °C), resulted in dramatically improved peak shape for the problem analytes (Figure 1).

Analysis of as many opioids as possible in a single extract has several advantages, in addition to the obvious savings in cost and time. Potent minor active metabolites of codeine, hydrocodone, and oxycodone produced by Cytochrome P450 2D6 metabolism (morphine, hydromorphone, and oxymorphone, respectively[6]) can be monitored routinely in this procedure. Information on potent active metabolites may be helpful in assessing total opiate exposure, and may also help to differentiate acute and chronic drug exposures. Using this LC/MS method, hydromorphone can be detected in three different kinds of cases: (1) after hydromorphone administration, (2) as a potent minor

Table 3. Method Limits of Detection, Quantitation, Linearity, and Quality Control Data

Compound	LOD ng/mL	LOQ ng/mL	Upper LOL	Control conc.	CV%
Ethyl morphine (I.S.)	-	-	-	-	-
Morphine	5	5	2000	41 92	10% 7%
Hydromorphone	1	2	400	8	8%
Codeine	5	5	2000	49 97	6% 5%
Oxycodone	5	5	2000	45 258	6% 4%
6-acetyl morphine	1	2	200	4	6%
Hydrocodone	5	5	2000	52 94	4% 6%
Benzoylcegonine	25	100	5000	114 672	10% 8%
Cocaine	5	5	2000	61 84	7% 6%
Cocaethylene	5	5	2000	64 88	11% 8%

metabolite of hydrocodone, and (3) as a minor metabolite after high-dose morphine administration[7].

We selected ethyl morphine as the internal standard for this method because some of the deuterated internal standards we tested fragmented to give the same ions as the homologous target compound in our single quadrupole instrument. If this method were employed with a tandem LC/MS system, multiple deuterated internal standards could be employed, which might result in even better accuracy and precision than reported here.

Oxymorphone and morphine-3- and morphine-6-glucuronides have only been analyzed on a research basis to date. Despite poor recovery, measurement of morphine-3- and morphine-6-glucuronides along with morphine appears to be valuable in differentiating some cases of acute vs. chronic drug ingestion. In one morphine-related death, a teenager diverted and took an unknown dose of an older woman's prescribed continuous-release morphine. Analysis by LC/MS revealed a post-mortem blood morphine concentration in

excess of 700 ng/mL, but lower concentrations of morphine glucuronides. In contrast, post-mortem blood from terminal cancer patients receiving chronic morphine typically contains morphine glucuronide concentrations on an order of magnitude greater than the parent drug concentration. Improved recovery of morphine glucuronides can be achieved by increasing the proportion of isopropanol in the eluting solvent in this method. Use of a simpler extraction with a hydrophobic solid-phase extraction column[2], rather than the mixed hydrophobic/cation-exchange column described in this method, gives excellent recovery of morphine glucuronides, but at the cost of increased background signal and shorter column life. An alternative extraction that may hold promise employs a polymeric solid phase column and elution with 5% ammonium hydroxide in methanol, with high recovery of morphine and its glucuronides[8].

Oxymorphone is extracted with high recovery in this method, and further work with oxymorphone is indicated because of its recent approval by the FDA as a high-potency oral opioid analgesic.[9] A number of other opioid metabolites can be mea-

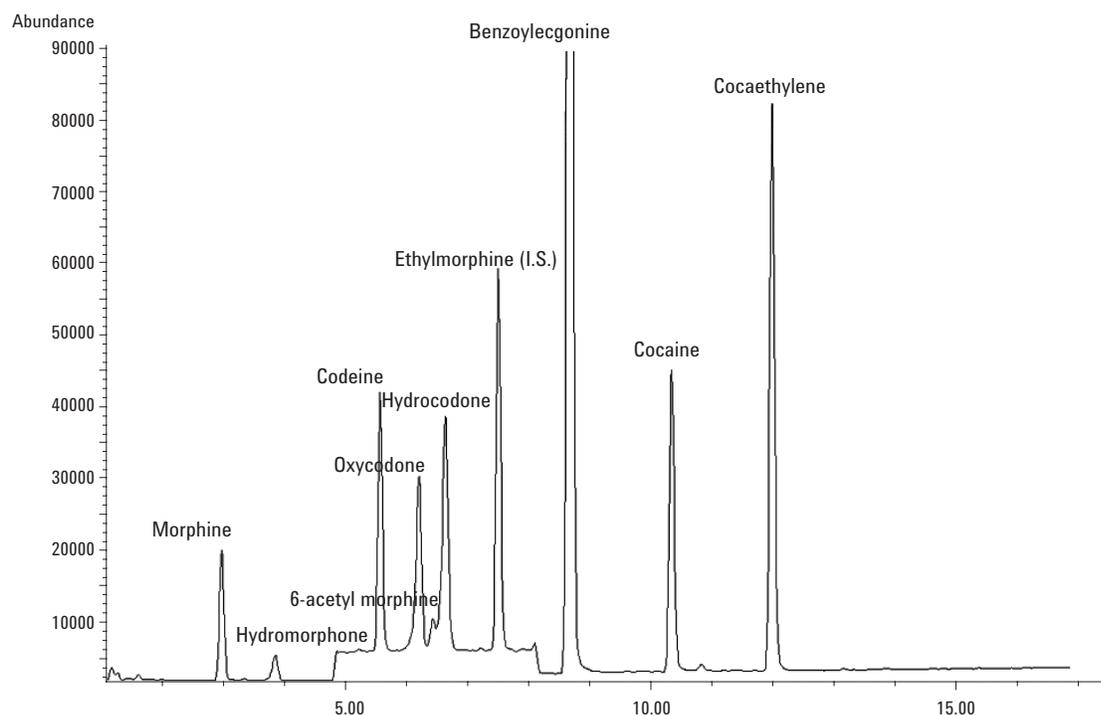


Figure 1. Total ion chromatogram of an extract of a quality control blood sample containing morphine (41 ng/mL), hydromorphone (8 ng/mL), codeine (49 ng/mL), oxycodone (45 ng/mL), 6-acetyl morphine (4 ng/mL), hydrocodone (52 ng/mL), benzoylcegonine (672 ng/mL), cocaine (61 ng/mL), and cocaethylene (64 ng/mL).

sured using this method. Hydrocodone is metabolized to hydromorphone, as previously noted, but is also metabolized to dihydrocodeine and norhydrocodone. Oxycodone is metabolized to oxymorphone, and is also metabolized to noroxycodone, alpha and beta oxycodol, noroxycodol, and other products. The choice of which metabolites to measure is complex. As mentioned previously, high-potency opioid metabolites may contribute to the effects of the parent drug, but recent data from Dr. Danny Shen's laboratory cast some doubt on this contention, at least with respect to oxycodone[10]. Even if metabolites do not contribute to the parent drug's pharmacological effects, they may be of toxicological interest, for example to help distinguish acute from chronic drug use.

Another cocaine metabolite, ecgonine methyl ester, was extracted with the solid phase extraction described in this paper, but recovery was variable, possibly due to losses during the evaporation step. Because of variable recovery, quantitative analysis of ecgonine methyl ester with this methodology would require use of a deuterated internal standard.

Another potential method enhancement to this method would be the use of the nebulizer shim (Agilent part number G1946-20307), which is designed to improve ion transit into the capillary when mobile phase flow rate is high. This could result in improved assay sensitivity for this application, which uses a mobile phase flow rate of 1.0 mL/min.

Conclusions

This communication describes a comprehensive method for analysis of opioids, cocaine, and cocaine metabolites in blood, using single quadrupole LC/MS with electrospray ionization after mixed-mode solid phase extraction. The method is superior to our previous GC/MS methodology in that derivatization is not needed, limits of detection and quantitation are lower, and a broader range of opioids can be detected. In addition, by using high-performance ZORBAX Eclipse Plus C18 HPLC columns at a relatively high temperature, we were able to eliminate previously encountered problems with poor opioid peak shape.

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