

Determination of Opiates and Metabolites in Blood Using Electrospray LC/MS

Application Note

Forensic Toxicology

Scott A. Schlueter and James D. Hutchison, Jr.
Montana Department of Justice, Division of Forensic Science

John M. Hughes
Agilent Technologies

Introduction

Opiates (Figure 1) are a widely abused class of drugs. The first metabolite of heroin is 6-monoacetylmorphine. It is commonly analyzed as a distinguishing marker of heroin use after an opiate-positive screening result. The well-established GC/MS method for the analysis of opiates¹ requires derivatization of these compounds. Derivatization adds variables to the analysis and can introduce aggressive derivatizing reagents into the analytical system.

Opiates and their metabolites are basic compounds that show excellent sensitivity in electrospray mass spectrometry, and can be analyzed without derivatization. The same solid-phase extraction (SPE) developed for the LC/MS analysis of plasma for clinical studies² can be used for the analysis of whole blood in forensic toxicology samples. The levels of opiates found in forensic blood samples are normally high enough that the scanning mode of data acquisition can be used

instead of selected ion monitoring (SIM). This allows other drugs isolated using the same sample preparation to be qualitatively identified in the same run that quantitates the opiates. The electrospray LC/MS analysis using scan mode gives accuracy and precision comparable to or better than those obtained using SIM in GC/MS.

Experimental

The Agilent 1100 Series LC/MS system included a binary pump, vacuum degasser, autosampler, thermostatted column compartment, diode-array detector, and the LC/MSD VL quadrupole mass spectrometer. The LC/MSD was used with an electrospray ionization (ESI) source. The diode-array detector was used primarily for method development purposes, although the UV spectral data obtained simultaneously with the MS data can be used for confirmation of identity of many drugs. Complete system control and data evaluation were carried out using the Agilent LC/MS ChemStation software.



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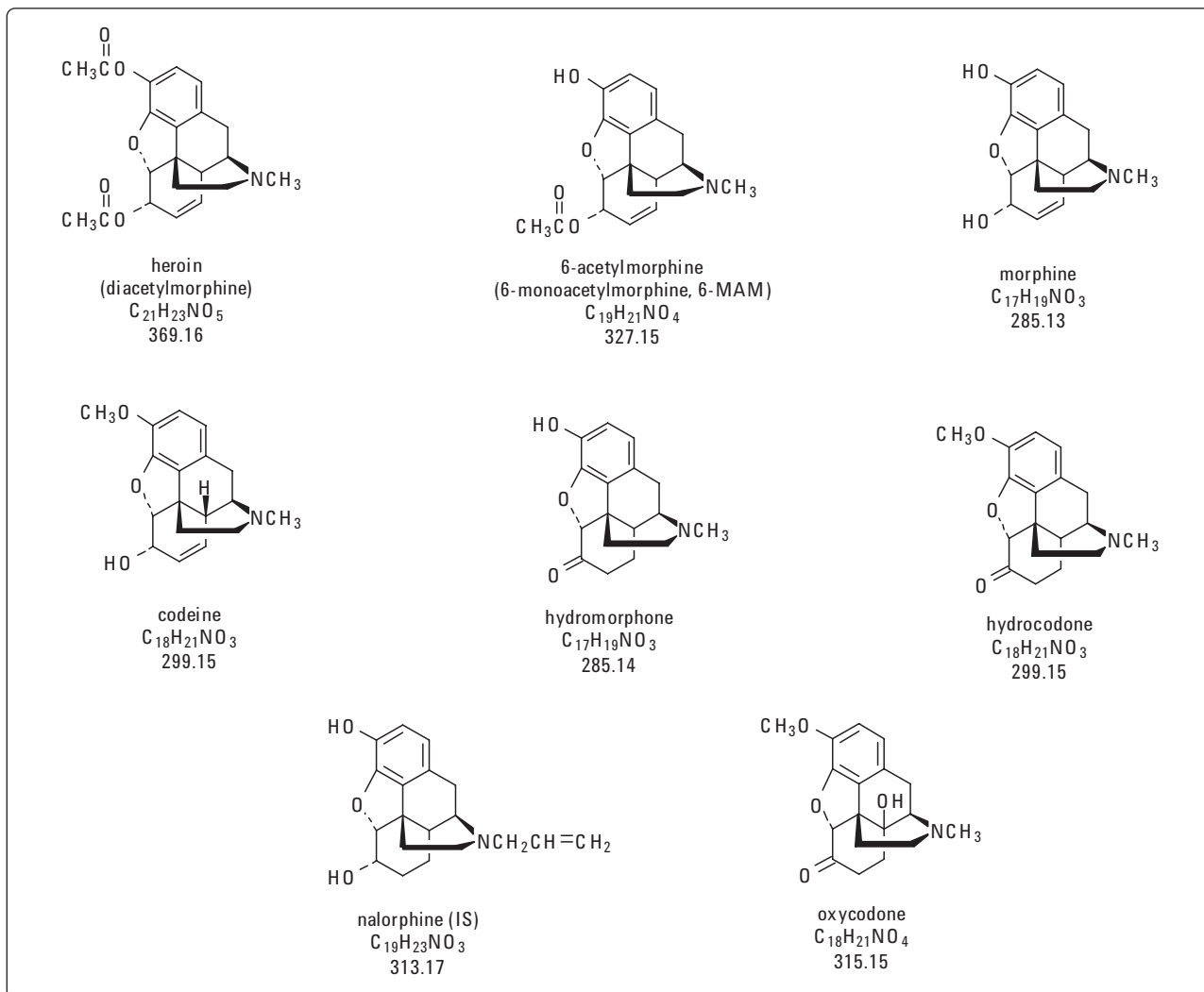


Figure 1. Opiate and internal standard structures

Analytical standards were obtained from Ceriliant Corporation (formerly Radian Analytical Products). The objective of developing a qualitative as well as a quantitative method mandated that the procedure use a non-deuterated internal standard. Nalorphine was chosen because the laboratory already had a validated protocol for opiates by GC/MS that used this internal standard.

Drug-free blood was fortified with known concentrations of the analytes and the internal standard. The tubes were capped, mixed, and incubated at 37°C for 12 hours. The sample blood (1 ml) was

spiked with internal standard (to 1 mg/l), mixed, and allowed to equilibrate for 30 minutes. A 2 ml aliquot of 10 mM ammonium carbonate buffer, pH 9, was added to each sample. The samples were then mixed again and centrifuged at 3000 rpm for 10 minutes.

Clean-up SPE columns (CEC18156, United Chemical Technologies) were conditioned with 2 ml of methanol and 2 ml of deionized water, followed by 2 ml of the ammonium carbonate buffer. The supernatant was transferred to an SPE column and allowed to pass through the conditioned

column by gravity flow. The column was rinsed with 2 ml of ammonium carbonate buffer. The column bed was dried at full vacuum for five minutes, and the analytes were eluted with 3 ml of methanol using gravity flow. The eluate was evaporated to dryness with a stream of nitrogen at 40°C. The final sample residue was reconstituted in 50 µl of LC mobile phase, transferred to a 1-ml microcentrifuge tube, and centrifuged at 15,000 rpm for 2 minutes. A 10 µl aliquot was then injected for analysis by LC/MS.

It should be noted that both morphine-3 glucuronide and morphine-6 glucuronide extracted favorably with this procedure. However, because the availability of commercial standards of the various opiate glucuronide conjugates is extremely limited, hydrolysis is a potential pretreatment option. A 1 ml aliquot of blood can be treated with 100 µl of a 10000-units/ml solution (pH 4.5) of β-glucuronidase isolated from *Patella vulgata*. For analysis of unknowns, the laboratory's standard operating procedure is to hydrolyze samples if presumptive screens indicated the presence of either opiates or benzodiazepines.

In the analysis of opiates, it is important to be able to clearly distinguish the isobaric molecules (morphine/hydromorphone, codeine/hydrocodone) for accurate interpretation of results. The chromatography for this method was therefore optimized to cleanly separate the various opiates in a reasonable time. This required gradient, rather than isocratic, conditions. The column could nonetheless be re-equilibrated quickly and retention times were extremely reproducible over time.

MS parameters optimized for this analysis included fragmentor voltage (to give the most intense protonated molecule for each analyte), capillary voltage (for maximum signal), and spray chamber parameters (for maximum signal with minimum noise).

ANALYSIS METHOD

Chromatographic Conditions

| | |
|-----------------------|---|
| Column: | Supelco Discovery HSC18, 4.6 mm x 15 cm, 3 µm |
| Mobile phase: | A = 0.1% formic acid in water B = methanol |
| Gradient: | Start with 5% B at 2 min 5% B at 10 min 90% B at 20 min 90% B |
| Flow rate: | 0.5 ml/min |
| Column temp: | 50°C |
| Injection vol: | 10 µl |
| Diode-array detector: | Signal 214, 8 nm; reference 360, 100 nm (used for method development only) |

MS Conditions

| | |
|--|-------------|
| Source: | ESI |
| Ionization mode: | Positive |
| Vcap: | 3000 V |
| Nebulizer: | 40 psig |
| Drying gas flow: | 13 l/min |
| Drying gas temp: | 350°C |
| Mass range: | m/z 100–650 |
| Fragmentor: | 120 V |
| Stepsize: | 0.1 |
| Peak width: | 0.12 min |
| Time filter: | On |
| Ions used for identification and quantitation: | |
| Nalorphine (IS) | m/z 312 |
| Morphine, hydromorphone | m/z 286 |
| Codeine, hydrocodone | m/z 300 |
| 6-Acetylmorphine | m/z 328 |
| Oxycodone | m/z 298 |

Results and Discussion

Recoveries for the analytes were excellent, ranging from a low of 85% for 6-acetylmorphine to a high of 100% for morphine. Figure 2 shows extracted ion chromatograms for the six opiates and the internal standard.

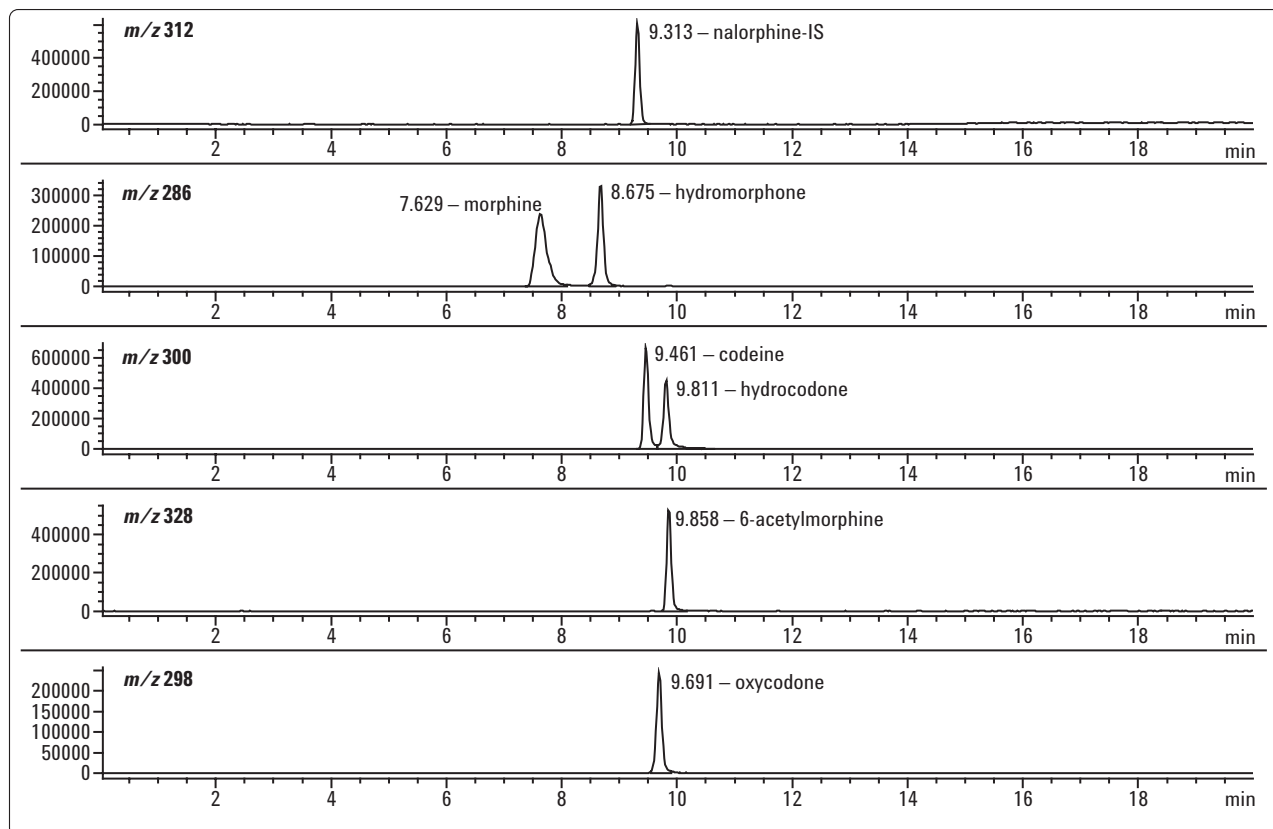


Figure 2. Extracted ion chromatograms (EICs) of opiates and internal standard

Figure 3 shows extracted ion chromatograms (EICs) for blank blood fortified with the internal standard at 1 mg/l (1000 ng/ml). Figure 4 shows extracted ion chromatograms (EICs) of control blood fortified with analytes at 0.25 mg/l (250 ng/ml).

The calibration range used for this analysis was 0.05–0.75 mg/l for all analytes. The calibration curves were linear across the calibration range without special weighting or curve treatment.

Typical calibration curves for the six analytes gave correlation coefficients (r^2) greater than 0.99 in all cases.

Quality control samples (n=10) fortified with 0.25 mg/l of each analyte gave quantitation results shown in Table 1. Coefficients of variation were typically 5% or less, and quantitation results were within 5% of the target value (within 1% or less for four of the analytes).

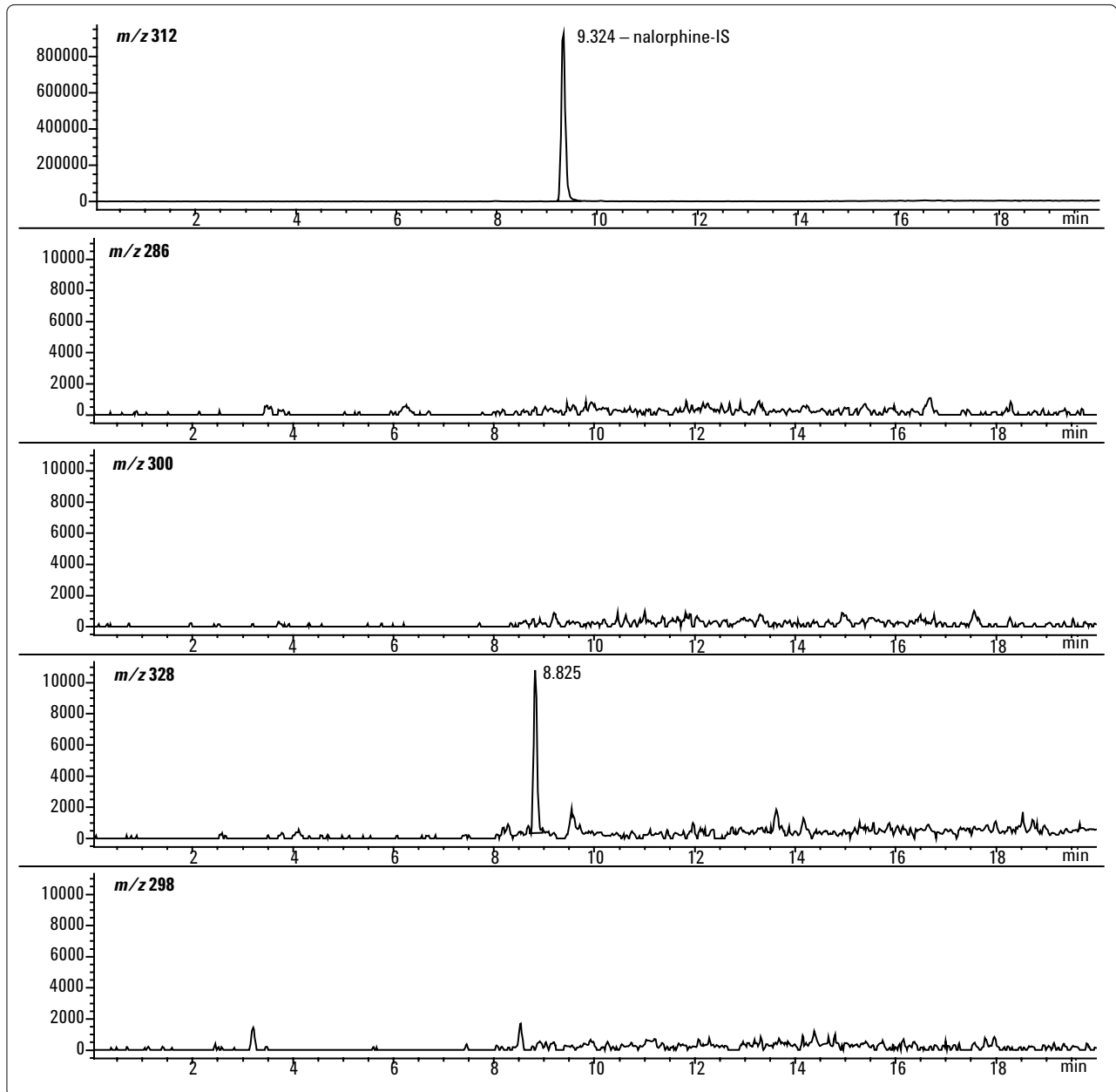


Figure 3. Extracted ion chromatograms (EICs) of blank blood fortified with internal standard at 1 mg/l (1000 ng/ml)

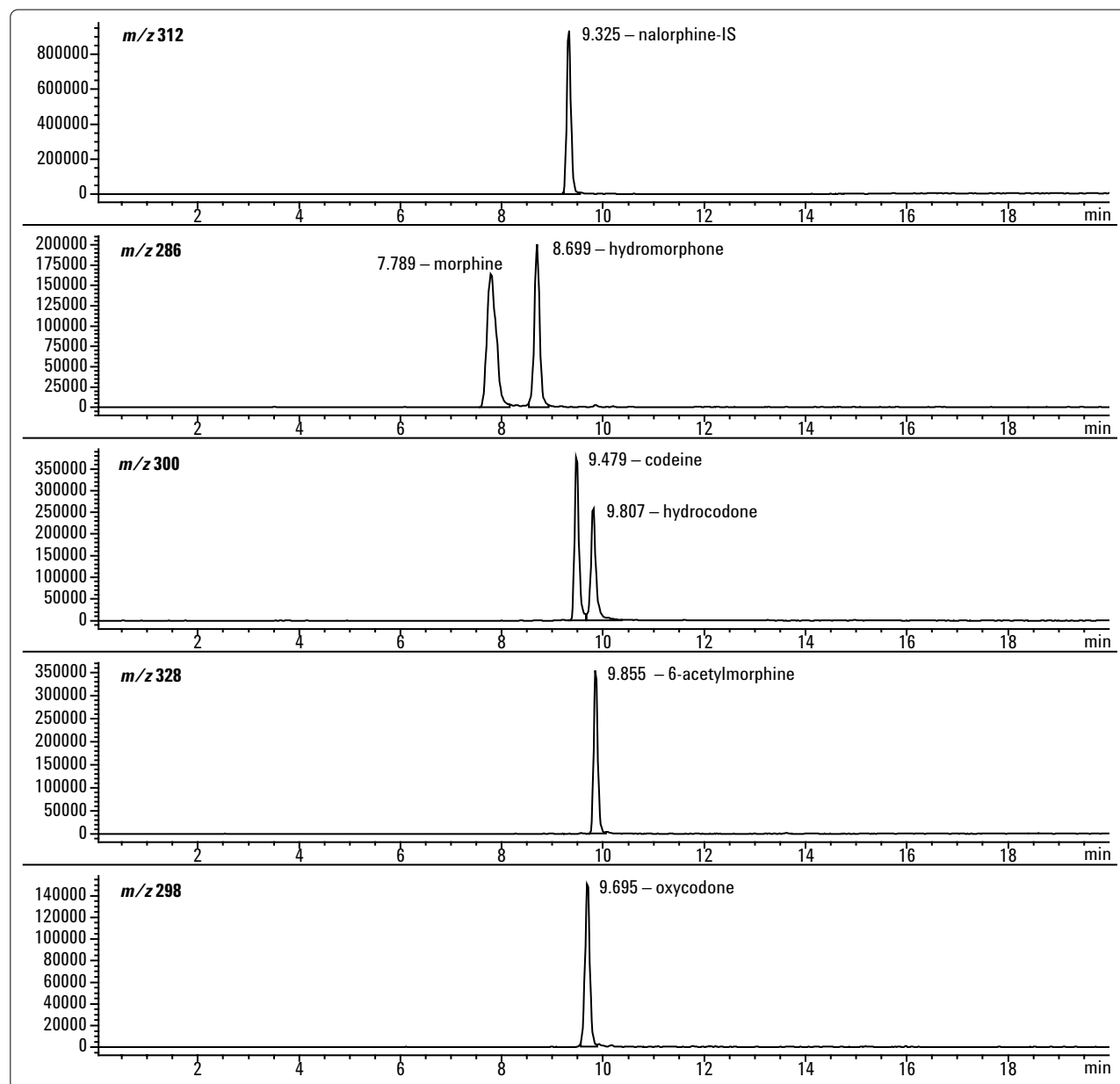


Figure 4. Extracted ion chromatograms (EICs) of control blood fortified with analytes at 0.25 mg/l

Table 1. Method accuracy and precision. Target concentrations were 0.25 mg/l

| | morphine | hydromorphone | codeine | hydrocodone | 6mam | oxycodone |
|---|-----------------|----------------------|----------------|--------------------|-------------|------------------|
| | 0.248 | 0.254 | 0.251 | 0.239 | 0.253 | 0.247 |
| | 0.247 | 0.245 | 0.239 | 0.242 | 0.249 | 0.254 |
| | 0.250 | 0.254 | 0.269 | 0.252 | 0.260 | 0.275 |
| | 0.267 | 0.249 | 0.246 | 0.245 | 0.230 | 0.264 |
| | 0.254 | 0.242 | 0.252 | 0.245 | 0.244 | 0.257 |
| | 0.251 | 0.246 | 0.249 | 0.241 | 0.248 | 0.267 |
| | 0.247 | 0.251 | 0.245 | 0.237 | 0.255 | 0.252 |
| | 0.258 | 0.259 | 0.256 | 0.250 | 0.258 | 0.263 |
| | 0.249 | 0.253 | 0.244 | 0.246 | 0.252 | 0.263 |
| | 0.254 | 0.256 | 0.261 | 0.249 | 0.259 | 0.287 |
| mean | 0.253 | 0.251 | 0.251 | 0.245 | 0.251 | 0.263 |
| standard deviation | 0.00942 | 0.00436 | 0.0128 | 0.00557 | 0.0128 | 0.0122 |
| coefficient of variation¹ | 3.729 | 1.737 | 5.102 | 2.276 | 5.117 | 4.638 |
| percent error² | 1.00% | 0.36% | 0.48% | -2.16% | 0.32% | 5.16% |

¹Coefficient of variation = (standard deviation/mean) x 100;

²percent error = (mean-target)/target x 100

Figure 5 shows the results for an opiate-positive blood sample from a deceased 48-year-old female. She had an extensive medical history and had recently been assigned prescriptions of MS Contin (morphine sulfate) and Dilaudid. Analysis confirmed the presence of total morphine at 0.84 mg/l and total hydromorphone at 0.08 mg/l.

Another positive blood sample (Figure 6) was from a case involving a 40-year-old male discovered unconscious with oxycontin in his pocket. He suffered from hepatitis C and had a history of drug abuse. LC/MS analysis of the subject's blood was positive for oxycodone at 0.23 mg/l and for codeine, which was not quantified because it was below the low calibrator (0.05 mg/l).

Analysis is also shown (Figure 7) for a third positive blood sample from a deceased 41-year-old female. A syringe and small packages of narcotics were discovered in the toilet. LC/MS analysis confirmed the presence of total morphine at 0.05mg/l, and clearly identified both 6-acetylmorphine and codeine at levels below the low calibrator.

The definition of the LOQs for this method is still in progress, but the sensitivity of the method reported here affords reliable quantitation down to at least 0.01 mg/l (10 ng/ml).

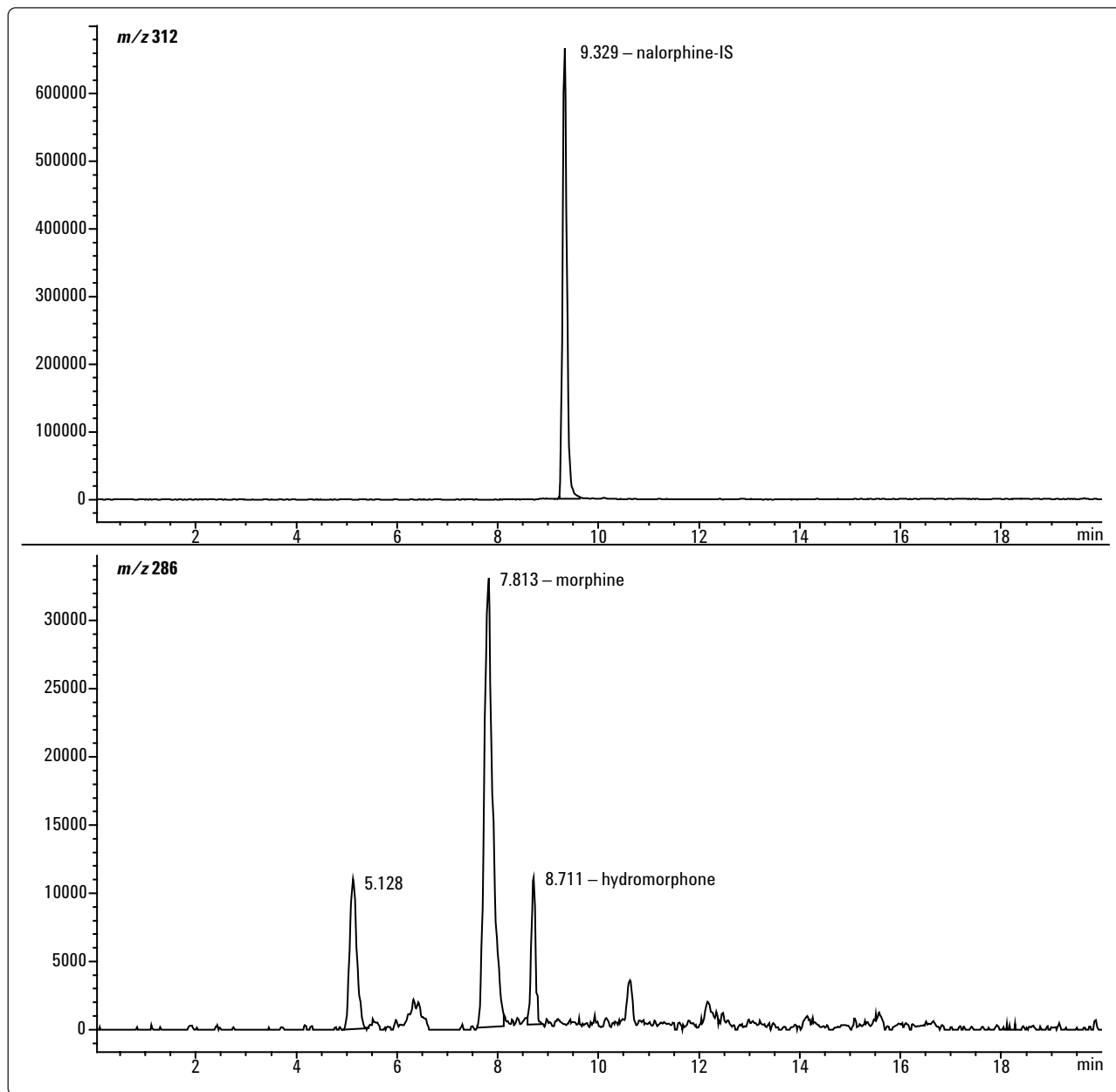


Figure 5. EICs of a positive blood sample found to contain morphine and hydromorphone

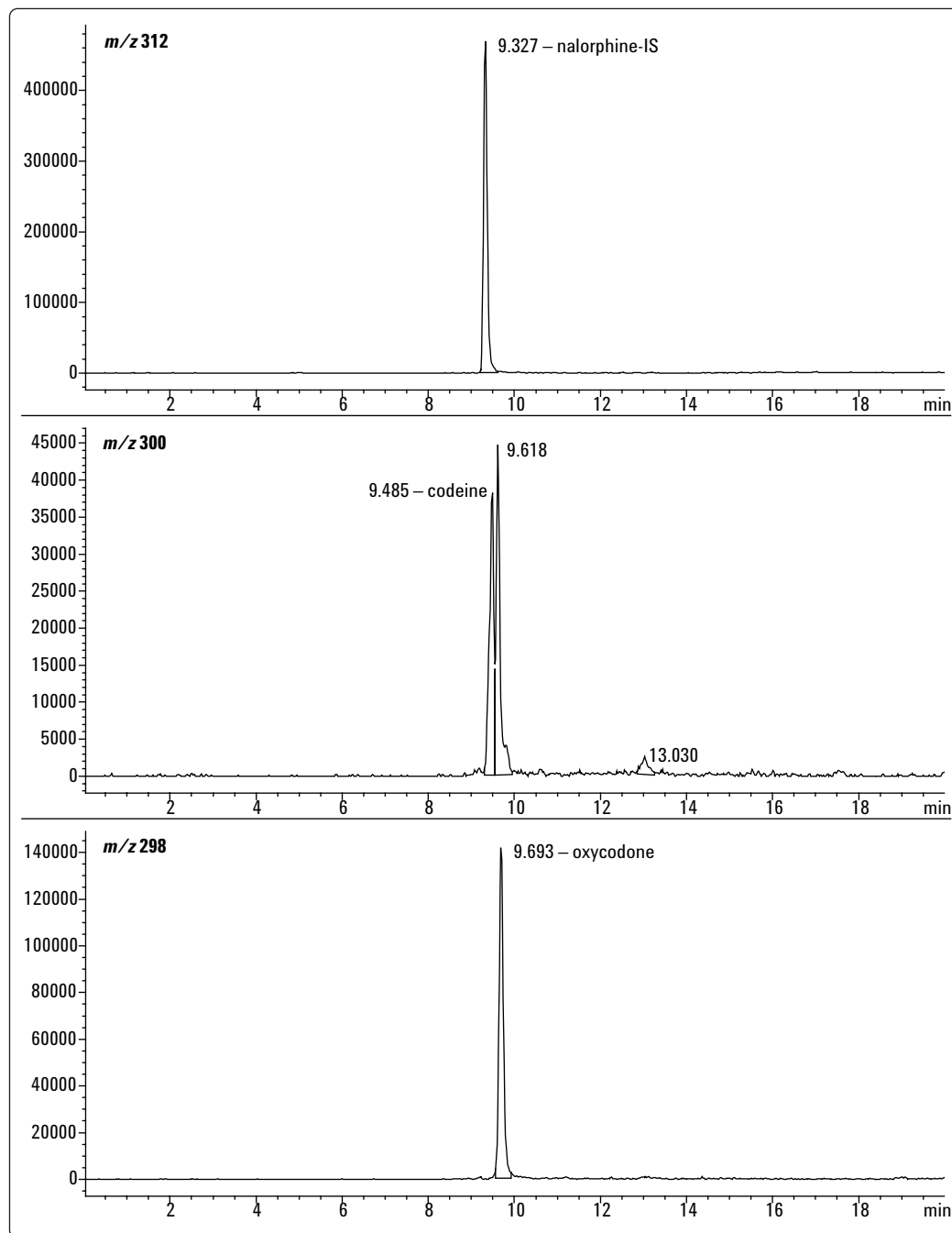


Figure 6. EICs of a positive blood sample found to contain oxycodone and codeine

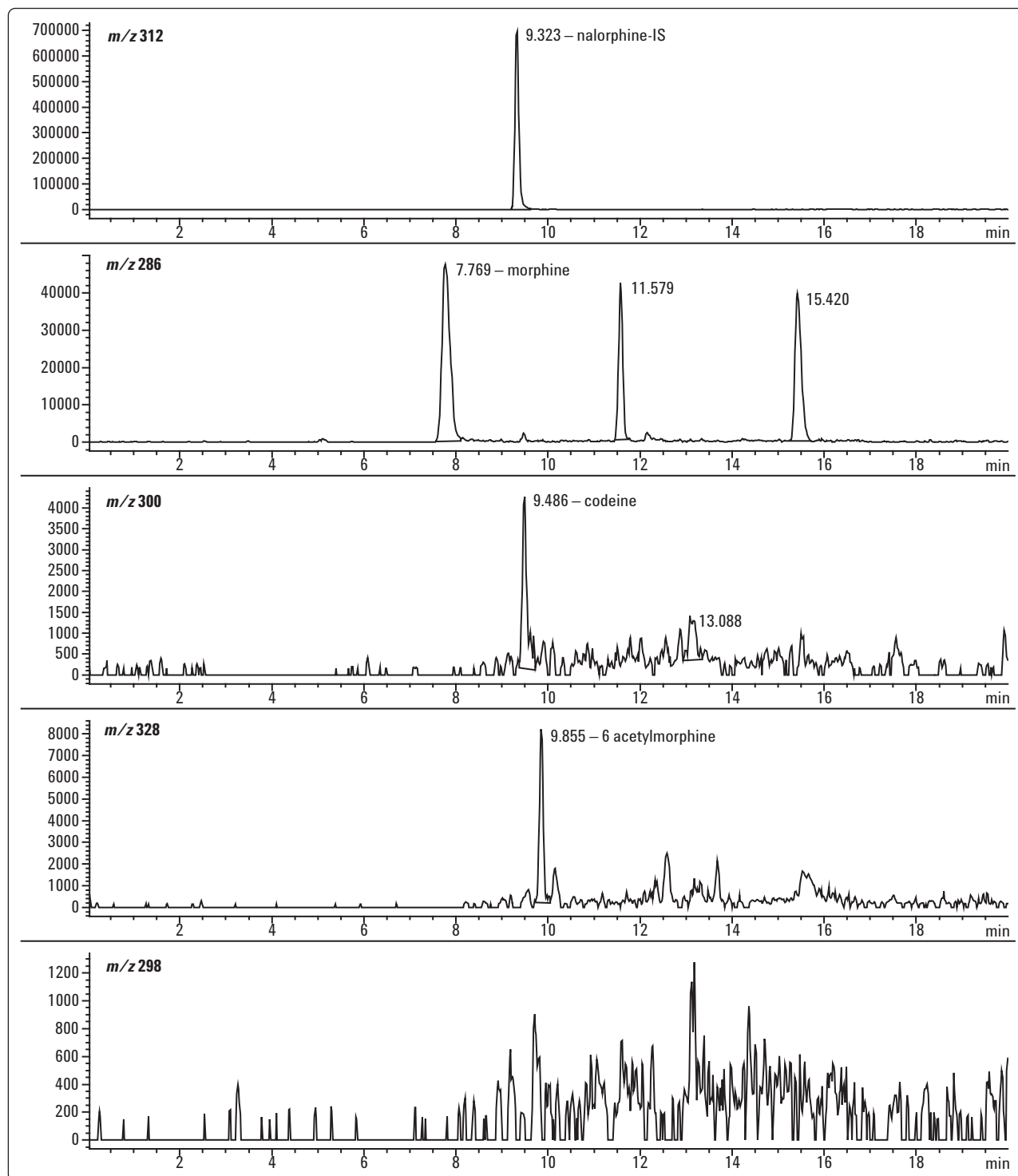


Figure 7. EICs of a positive blood sample found to contain morphine, codeine, and 6-acetylmorphine

Conclusions

The data clearly show the described electrospray LC/MS method to be suitable for routine measurements of opiates in whole blood. The reproducibility of the assay thus far has a linear range of 0.05–0.75 mg/l, and the precision and accuracy of this method compare favorably to those of the well-established GC/MS methods for forensic drugs in blood. The sample preparation uses a solid phase extraction technology widely used in forensic laboratories and requires no special modifications. In comparison to an existing GC/MS method for these analytes, the LC/MS method is simpler because it does not require derivatization, which involves aggressive reagents, derivatization time, and additional variability. In addition, the sensitivity of the LC/MSD VL allows the use of scan mode rather than SIM without compromising accuracy or precision, making this method useful for general drug screening as well as target compound analysis.

References

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2. Matthew H. Slawson, Dennis J. Crouch, David M. Andrenyak, Douglas E. Rollins, Jeffrey K. Lu, and Peter L. Bailey, Determination of Morphine, Morphine-3-glucuronide, and Morphine-6-glucuronide in Plasma after Intravenous and Intrathecal Morphine Administration Using HPLC with Electrospray Ionization and Tandem Mass Spectrometry. *Journal of Analytical Toxicology*, 23, 468–473 (1999).

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Authors

Scott Schlueter and **Jim Hutchison** are ABFT-certified Forensic Toxicologists at the Montana State Crime Lab.

John Hughes is an Applications Chemist at Agilent Technologies.

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