# Certification of Reference Materials: Purity Analysis of Morphine-3β-D-Glucuronide by Quantitative NMR, Enzymatic Hydrolysis LC-MS/MS Assay, and Mass Balance Purity Factor

Huahua Jian, Martha Liu, Uma Sreenivasan and Isil Dilek
Cerilliant Corporation, Round Rock, Texas 78665, USA

## Introduction and Objective

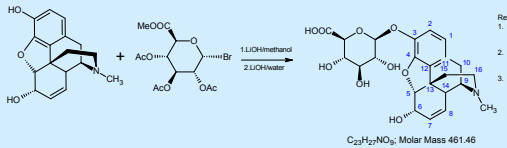
Many drugs are rapidly and extensively metabolized to their glucuronide conjugates for excretion. Detection and quantitation of drug glucuronides is an essential part of forensic and toxicologic analyses with potential social, legal and clinical significance. The purity value assigned to these reference materials therefore directly affects the quality of analytical results.

As a class, glucuronides tend to be polar, hygroscopic, difficult to manufacture, handle, and certify. A comparative evaluation of different approaches to certification of drug reference materials was performed using Morphine-3β-D-glucuronide (M3G) as an example.

## Materials and Methods

M3G was synthesized at Cerilliant Corporation. The purity of M3G was determined by three methods: 1. Mass balance/purity factor analysis by adjusting for chromatographic purity, residual water, residual inorganic and residual volatile organic content;
2. Hydrolysis of M3G by glucuronidase enzyme followed by assay using LC-MS/MS traceable to a morphine reference standard;
3. Quantitative $^1$H NMR (qNMR) analysis by comparison to a NIST-traceable universal reference standard

## Synthesis of Morphine-3-D-Glucuronide




$C_{23}H_{27}NO_9$; Molar Mass 461.46

References:
1. B. Berrang, C. E. Twine, G. L. Hennessee and F. I. Carroll. *Synth. Commun.*, 1975, **5, 221.**
2. H. Yoshimura, K. Oguri and H. Tsukamoto, *Chem. Pharm. Bull.*, 1968, **16, 214.**
3. H. Yoshimura, M. Mori and K. Oguri, *Chem. Pharm. Bull.*, 1970, **18, 2548.**

## 1. Mass Balance Purity Factor

$$PurityFactor = \left[[100-(wt\%Solvents)-(wt\%H_2O)-(wt\%Inorganics)]*\frac{ChromPurity}{100}\right]$$

**wt%Solvents:** the weight percentage of residual solvents present in the neat material, determined by GC/FID-headspace injection = **None Detected.**
**wt%$H_2O$:** the weight percentage of water present in the neat material, determined by Karl Fischer coulometry = **4.95%.**
**wt%ROI:** the weight percentage of inorganic content in the neat material, determined by sulfated ash analysis = **Below Quantitation Limit.**
**ChromPurity**: based on the chromatographic purity of the specified primary purity method, determined by average of two HPLC methods = **99.475%.**

**Purity Factor = 94.55%**

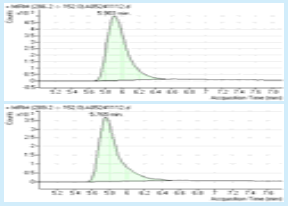
**Discussion:** Mass balance purity factor analysis requires complete characterization of the material. All analyses must be accurate to provide an accurate purity factor value. For a hygroscopic material such as M3G, material storage & handling can alter the residual $H_2O$% result. Exposure of M3G to varying conditions over 2-3 weeks resulted in 4.6% to 12.4% residual $H_2O$% content demonstrating the need to re-evaluate water content before each use.

## 2. Enzymatic Hydrolysis LC-MS/MS Assay


**Enzyme Hydrolysis Conditions:**
Morphine calibration curve was prepared from certified morphine solution standard (Cerilliant M-018). Morphine-d3 (Cerilliant, M-006) was added as internal standard (ITSD) for signal normalization. The curve points and three assay solutions were incubated with β-glucuronidase from *Helix Pomatia* (Sigma, G7017) with pH 4.7 buffer. The hydrolyzed sample was filtered, diluted and analyzed by LS-MS/MS on an Agilent G6410 triple quad system.

**LS-MS/MS Quantitation**

| | | | |
|---|---|---|---|
| Column: | Luna 3u C18(2) 100A, 100*2.00 mm; Column temperature: ambient | | |
| Mobile Phase: | A: 0.1% formic acid/methanol<br>B: 0.1% formic acid/water | | |
| Gradient: | Time (min) | A% | B% |
| | 0 | 5 | 95 |
| | 3 | 60 | 40 |
| | 7 | 60 | 40 |
| | 7.1 | 95 | 5 |
| | 10 | 95 | 5 |
| Flow Rate: | 0.3mL/min | Injection Vol. | 3 µL |
| ESI Source Parameters : | Drying Gas Flow 13 L/min; Nebulizer Pressure: 60 psig; Drying Gas Temperature: 350 °C; Capillary Voltage: 4000 V. | | |
| Quantitation | MRM (Positive); Fragmentor = 110V; Collision energy = 75V | | |
| | Precursor Ion | Product Ion | |
| Morphine | 286.2 | 152.0 | Quantifier |
| | 286.2 | 165.0 | Qualifier |
| Morphine-$d_3$ | 289.2 | 152.0 | Quantifier |
| | 289.2 | 165.0 | Qualifier |



| P. | Prep 1 | Prep 2 | Prep 3 | |
|---|---|---|---|---|
| | 58.135 | 58.110 | 59.810 | |
| | 57.559 | 58.249 | 59.247 | |
| | 57.754 | 58.507 | 58.654 | |
| Avg | 57.816 | 58.289 | 59.237 | 58.447 |
| RSD | 0.506% | 0.346% | 0.976% | 1.219% |

| | |
|---|---|
| Morphine M.W. | 285.34 |
| M3G M.W. | 461.40 |
| M3G prepared concentration | 105.80 |
| **M3G assay** | **89.33%** |

**Discussion**: Enzyme hydrolysis assay is an indirect method of analysis. Caution is required to ensure method specificity. Impurities in M3G, e.g. residual morphine (synthetic precursor) could bias results. This lot of M3G contained no morphine by HPLC. In addition, after hydrolysis, impurities were investigated by LCMS to ensure that M3G hydrolyzed exclusively to morphine in the assay analysis. Not every glucuronide can be certified by this method.

## 3. Quantitative 1H-NMR

In quantitative NMR (qNMR), the intensity of a signal is proportional to the number of nuclei observed under the signal. By adding a known amount of standard into known amount of analyte, the purity of the analyte can be calculated from the purity of the standard.

M3G and the internal standard, 3,5-dinitro benzoic acid (Fluka TraceCert®, 15636) were accurately weighed on a Mettler Toledo XP56 (6-place) balance. Six samples were prepared.

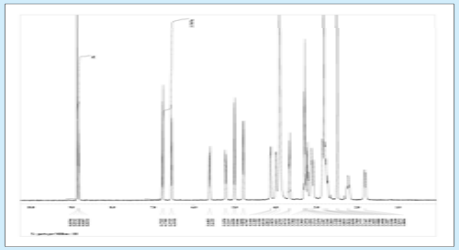
$$P_A = \frac{I_A}{I_{std}} \times \frac{n_{std}}{n_A} \times \frac{m_{std}}{m_A} \times \frac{M_A}{M_{std}} \times P_{std}$$

*P* – Purity;
*I* – Integer of signal;
*n* – Number of protons under the signal of interest;
m – Mass of component;
M – Molecular weight.
*std* – Internal Standard.
*A* – Component whose purity needs to be calculated.

**A:** Morphine-3β-Glucuronide
**ITSD:** 3,5-Dinitrobenzoic Acid

| | | | | | |
|---|---|---|---|---|---|
| | *Istd* = | 3000 | | *MA* = | 461.40 |
| | *nstd* = | 3 | | *Mstd* = | 212.12 |
| | *nA* = | 2 | | *Pstd* = | 99.43 |
| | *mstd* = | 14.810 | | *Vstock* = | 10.00 |

| Sample | FRN | *IA* | *Vsample* | *mA* | *PA* |
|---|---|---|---|---|---|
| # 1 | Q05061109-12 | 3279.126 | 1.700 | 9.772 | 91.361 |
| # 2 | Q05061110-23 | 3044.792 | 1.700 | 9.049 | 91.610 |
| # 3 | Q05061111-21 | 3682.254 | 1.700 | 11.298 | 88.736 |
| # 4 | Q05061112-26 | 3785.862 | 1.700 | 11.277 | 91.403 |
| # 5 | Q06061113-25 | 3664.359 | 1.700 | 11.166 | 89.349 |
| # 6 | Q05061114-24 | 5393.816 | 1.000 | 9.469 | 91.228 |
| | | | | RSD% | 1.37% |
| | | | | qNMR Average | 90.615 |



**NMR Experimental Conditions:**
JEOL ECS-400 spectrometer (399.7822 MHz for $^1$H); JEOL 40TH5AT / FG 2D probe; NMR solvent: DMSO-$d_6$:$D_2O$ = 80:20; Single pulse experiment with 1H detection; Pulse angle 30°; Relaxation delay 50s; Scan number 32 with 2 pre-scan; Spin state OFF. Auto receiver gain; Sweep offset 6ppm; sweep width 36ppm. Post transformation: manual phase adjustment; Reference to solvent peak. Monitor signals: 2 aromatic signals for M3G, 3 aromatic signals for ITSD.

**Discussion:**
Sample weighing is a critical step in qNMR analysis – a 6 or 7 place balance is required. Quantitative NMR is a good orthogonal method to certify reference materials for quantative applications.

## Results

- Morphine-3β-D-glucuronide (M3G) reference material with high chromatographic purity (99.5%) was used to compare different methods of reference material certification. The difference in results between techniques was shown to be due to hygroscopicity of the neat reference material at different time points. This illustrates the importance of re-evaluation of water content for hygroscopic materials before each use.
- Quantitative NMR is a powerful tool for quantitation provided attention is paid to sample preparation, weighing and solubility.
- LS-MS/MS is suitable when impurity profile and specificity are considered and a suitable reference material is available as calibrator.
- Purity assignment by LCMS assay and by qNMR do not provide details on impurity profile and chromatographic behavior, which are important considerations for test method performance.
- The mass balance purity factor approach does provide detailed information on chromatographic impurity profile and residual impurities. For a neat reference material, changes in residual water content over time can affect test results. Use of the purity factor with re-evaluation of water content before each use provides an accurate and complete characterization of the reference material.

## Conclusion

- Three approaches presented for analysis of M3G purity. The approaches are applicable to a variety of reference materials.
- The reference material purity assignment must take into consideration sample handling, stability, hygroscopicity and chromatographic properties.
- In evaluating reference materials and their suitability for an intended purpose, it is important to understand how the purity value was assigned. Material properties, such as hygroscopicity, are also an important consideration when using or certifying neat reference materials in the lab.

Cerilliant® Analytical Reference Standards a SIGMA-ALDRICH company