Quantitative Analysis of Morphine in Urine by Gas Chromatography-Chemical Ionization-Mass Spectrometry, with [N-C$^{2}$H$_{3}$]Morphine as an Internal Standard

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A highly sensitive and specific method is described for quantitative determination of morphine in urine. Used in the method is a gas chromatograph and a computerized, quadrupole mass spectrometer operated under chemical ionization conditions. Morphine concentrations as low as 5 ng/ml can be measured by selected ion monitoring, with $N$-trideuteromethyl morphine as the internal standard. The sensitivity and specificity of the method is illustrated by the detection and quantitation of morphine in urine after ingestion of cough medicine containing codeine.

Hammar et al. (1) first described the application of mass fragmentography [also called multiple ion detection (2) or selected ion monitoring (3)] to the identification of chlorpromazine and its metabolites in blood. Strong and Atkinson (4) extended use of this method to lidocaine and its pharmacologically active metabolite, with use of trimercaine as an internal standard. The use of stable isotope-labeled internal standards has made selected ion monitoring particularly attractive for quantitative analysis of drugs in biological fluids. Gaffney et al. (5) utilized deuterated nortriptyline as an internal standard in a study of low concentrations of nortriptyline in plasma. With such deuterated internal standards, no corrections need be made for chemical differences between the drug and its internal standard.

We describe here the application of selected ion monitoring to the analysis of morphine in urine with a computer-controlled quadrupole mass spectrometer operated under chemical ionization conditions.

Materials and Methods
Deuterated Morphine

Morphine was converted to [N-C$^{2}$H$_{3}$]morphine for use as an internal standard by a previously described procedure (6, 7). Mass spectral analysis of the deuterated morphine showed that 94% of the molecules contained three deuterium atoms, while less than 0.5% contained no deuterium atoms. Gas chromatography indicated a chemical purity of greater than 99%.

Instrumentation

A Varian 1740 gas chromatograph was coupled directly to a Finnigan 1015 quadrupole mass spectrometer equipped with a chemical ionization source. Methane was used as the carrier gas and also served as the chemical ionization reactant gas (8). The gas chromatograph was fitted with a 2.0 m × 2 mm (i.d.) glass column packed with 3% OV-17 coated on 100/120 mesh Gas Chrom Q solid support (Applied Science Labs, State College, Pa.). The flow of methane through the column was 19 ml/min. The temperature of the injection port was 290 °C, of the column 230 °C, and of the transfer line oven 290 °C. The pressure in the mass spectrometer ion chamber was about 66 Pa (0.5 Torr). The ion source was operated at an ionizing energy of 100 eV, an ion repeller voltage of 0 V, and a filament emission of 300 μA.

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The mass spectrometer was interfaced to a Systems Industries 250 computer system, which controlled the scan of the mass spectrometer and processed and displayed the mass spectral data.

**Computer Programs**

The computer programs used to control the mass spectrometer and display the mass spectral data were developed at Battelle. The data-acquisition program allows selection of up to 8 ion masses from anywhere within the mass range of the mass spectrometer (1 to 750). The ion current at each of the masses to be monitored is integrated for a selectable length of time. To achieve a high signal to noise ratio, a least-squares smoothing operation is included in the program (9).

While data are being acquired, a real-time plot of the ion current of the first mass selected is displayed on a digital plotter. After data acquisition is completed, the signal-averaged and normalized ion currents for each of the selected ion masses are plotted. The individual ion current curves can be overlaid or displaced horizontally, to facilitate visual comparison.

**Preparation of Samples**

The procedure used to prepare urine samples for analysis is illustrated by the following example.

\[ [N-C^2H_3] \text{Morphine internal standard solution, 0.1 ml of a solution of 50 \mu g/ml in 1 molar HCl, was added to 10 ml of urine. The urine was buffered to} \]

\[ \text{pH 8.5 and 10 ml of chloroform-isopropanol (4:1 by vol) was added. After thorough mixing, the solution was centrifuged and the organic phase separated and evaporated under a stream of nitrogen. The sample residues were stored at 4}^\circ\text{C until just before analysis, at which point they were trimethylsilylated. The morphine in the urine extracts was quantitatively trimethylsilylated by adding 25 \mu l of bis(trimethylsilyl)acetamide (Pierce Chemical Co., Rockford, Ill.) to each extract, followed by warming at 60}^\circ\text{C for about 1 h. The resulting solution was either analyzed directly or diluted with chloroform (50 \mu l) and stored at 4}^\circ\text{C until analyzed.} \]

**Analytical Procedure**

About 2 \mu l of the trimethylsilylated solution of urine extract was injected into the gas chromatograph–mass spectrometer system. Under the chromatographic conditions used, the bis(trimethylsilyl)morphine and its deuterated analog had identical retention times of 3.3 min. At least 10 min was allowed between successive sample injections, to allow most other components of the urine to elute from the chromatographic column.

During injection of the sample the effluent from the column was vented to a rotary pump. About 1 min after injection, the column effluent was introduced into the mass spectrometer by switching a specially constructed (at Battelle) high-temperature, low-internal-volume, three-way valve. The ion-source filament was then turned on and data acquisition begun.

Under computer control the mass spectrometer sequentially monitored the ion currents at each of the selected masses for 170 ms, repeating the cycle until the bis(trimethylsilyl)morphine had eluted from the column (3.3 min). After each run was completed, we obtained computer plots for the ion currents at each of the selected masses, and determined the concentration of morphine in the urine by measuring the ratio of peak heights corresponding to silylated morphine and the deuterated internal standard (340/343 or 414/417), and multiplying this ratio times the slope of the standard curve (Figure 1).

**Results and Discussion**

To establish standard curves, we added known amounts of morphine and \([N-C^2H_3] \text{morphine to aliquots of morphine-free urine. The ion-current signals for the bis(trimethylsilyl)morphine and the deuterated internal standard are easiest to measure accurately if they are of comparable intensities. Consequently, known amounts of morphine were added to two sets of five 10-ml urine samples. One set covered morphine concentrations of 0 to 1000 ng/ml, the other 0 to 100 ng/ml. Five micrograms of} \([N-C^2H_3] \text{morphine was added to each of the samples in the first set; only 0.5 \mu g of the internal standard was added to each of the samples in the second set.} \]

In each analysis, four masses were monitored: \(m/e\) 340, 343, 414, and 417. They correspond to the prominent fragment ions \(M^+\)-\(HOSi(CH_3)_3\) and \(M-C^2H_3^+\) in the methane chemical ionization spectra of bis(trimethylsilyl)morphine and \([N-C^2H_3] \text{bis(trimethylsilyl)morphine (Figure 2). Although it was only necessary to monitor one pair of masses, monitoring an additional pair provided convenient corroborative information. Any large differences between the 340/343 and 414/417 ratios for the ion-current peaks within a given run was strong evidence for the contribution of another urine component to the ion current at one of the monitored masses. Interference by other components in the urine was not a frequent problem. When it did occur it was easily detected by this technique, and could be avoided by varying the conditions of gas-chromatography or by choosing another pair of ion masses to monitor.} \]

The standard curves (Figure 1) indicate that the measurement of morphine concentration is linear over the range studied, 5 to 1000 ng/ml. Our experience and reports of similar studies in which selected ion monitoring is done with isotope-labeled internal
To evaluate the reproducibility of the ion current ratio measurements, we injected five successive 1-μl aliquots of a solution containing equal amounts (100 ng/ml) of bis(trimethylsilyl)morphine and [N-C\textsubscript{2}H\textsubscript{3}]bis(trimethylsilyl)morphine. The standard deviation for the m/e 340/343 and 414/417 ion current peak height ratios amounted to 2.8 and 2.0%, respectively.

Although we consider the reported reproducibility adequate for most applications of this technique, it is not representative of the reproducibility that can be achieved. Probably the major limitation to the experimental reproducibility in this work was the short-term stability of the rf and dc voltages applied to the quadrupole rods determining the mass focus. For maximum sensitivity and reproducibility, the mass spectrometer should be focused on the exact center of the ion peak that it is monitoring. Any instability in the focusing voltages can result in the focus being offcentered, giving an inaccurate ion current measurement.

Also, we made no provision for mass-defect differences between the ions from the calibration compound (perfluorotributylamine) and the ions from the bis(trimethylsilyl)morphine. The m/e 414 ion from bis(trimethylsilyl)morphine has a calculated mass of 414.22, as compared to 413.98 for the isobaric ion from the calibration compound. The 0.3 amu (atomic mass unit) difference meant that the ion currents were being monitored at points on the sides of the ion peaks. A recent modification to our selected ion monitoring computer program avoids this problem by including a provision for specifying the masses to be monitored to the nearest 0.1 amu.

The advantages of selected ion monitoring have been amply described previously (13). Most notably, the technique is capable of very high specificity and sensitivity. Another compound can interfere only if it has the same gas-chromatographic retention time and generates ions at the same m/e values as the compound being analyzed. Because of the relative lack of interference, the full amplification power of the electron multiplier and associated amplifiers can be used. However, the degree of interference does depend on the uniqueness of the ion masses monitored. It is in this regard that chemical ionization offers significant advantages over the more commonly used electron impact ionization. Ideally, the ion current from the compound being analyzed is concentrated in a few relatively high m/e values where background contributions are low. This is precisely the situation encountered in the methane or isobutane chemical ionization mass spectra of most drugs (14). Chemical ionization has the additional advantage of eliminating the need for a separator between the gas chromatograph and the mass spectrometer. The separator can be a major source of sample loss, owing to adsorption, decomposition, or diffusion.

Because of day-to-day variations in the performance of gas chromatograph–mass spectrometer systems, it is necessary to use an internal standard if

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**Fig. 1.** Standard curves for quantitative determination of morphine in urine

The curves were prepared by analyzing urine samples to which known amounts of morphine and [N-C\textsubscript{2}H\textsubscript{3}]morphine were added.

**Fig. 2.** Methane chemical ionization mass spectra of bis-(trimethylsilyl)morphine (upper) and [N-C\textsubscript{2}H\textsubscript{3}]bis(trimethylsilyl)morphine (lower).
quantitative reliability is important. An isotopically labeled compound having the same structure as the molecule under study constitutes the ideal internal standard, because the two compounds will exhibit essentially identical chemical characteristics (5). Consequently, any losses resulting from incomplete extraction, derivative formation, or adsorption on the column will occur in direct proportion to their respective concentrations. Thus, the concentration of a drug can be easily and accurately determined when a known amount of isotopically labeled standard is added to the urine and the relative amounts of the drug and internal standard eluting from the column are measured by selective ion monitoring.

The major limitation to the use of stable isotope-labeled internal standards is their current unavailability. In most cases the isotope-labeled drug must be specially synthesized. Of course, the difficulty of the synthesis will vary greatly, depending on the structure of the drug and on the location and number of isotopic labels. Preferably, the radiolabeled drug will have a mass difference of at least three amu, to minimize interference by $^{13}$C-containing ions. Obviously, only those ions that retain the isotope label are useful for selective ion monitoring. This is not ordinarily a problem when chemical ionization is used because the fragmentation is relatively limited and predictable. However, when the meth-
ane chemical ionization mass spectra of bis(trimethylsilyl)morphine and its N-C$_2$H$_5$ analog (Figure 2) are compared, it is evident that the $m/e$ 371 fragment ion cannot be used for selective ion monitoring because its formation involves loss of the portion of the molecule containing the isotope label (C$_5$H$_7$NHC$_2$H$_5$).

An additional point worth noting regarding the system used in this study is that a computer-controlled quadrupole mass spectrometer is particularly well suited for the selective ion-monitoring technique. After the system has been mass calibrated by the normal procedure any one or more ion masses within the mass range of the instrument can be monitored by simply designating to the computer the $m/e$ values and integration times. Depending on the input/output devices and software available, the data can be presented in a variety of formats.

A practical test of the procedure. In view of wide interest in the detection and measurement of morphine in urine, we wished to compare this technique with current clinical methods. For this purpose, aliquots of urine samples obtained from 20 patients on a methadone maintenance program were analyzed by the procedure described here, and the data compared with parallel analyses by the other techniques (15). On the basis of these results, we concluded that the sensitivity of the selected ion monitoring technique is comparable to that of radioimmunoassay or hemagglutination analysis. However, the latter two techniques are more subject to cross interference from compounds having structures similar to morphine.

While this investigation was in progress, we were asked to analyze the urine of a suspected drug user. The results of this analysis illustrate the specificity and sensitivity of the specific ion-monitoring technique.

The submitted urine sample was adjusted to pH 9.3 and extracted with chloroform. The concentrated chloroform extract was analyzed by gas chromatography–chemical ionization mass spectrometry, with repetitive scanning over the mass range 100–400. In addition to nicotine, caffeine, and several endogenous compounds, a substantial peak corresponding to codeine was observed. The presence of codeine was consistent with the subject’s claim of having taken codeine-containing cough medicine the previous evening. To test specifically for morphine, we added 0.2 μg of [N-C$_3$H$_5$]morphine to 20 ml of urine and the sample was extracted at pH 9.3 with chloroform. The extract was trimethylsilylated and analyzed by specific ion monitoring as previously described. Under the chromatographic conditions used, bis(trimethylsilyl)morphine elutes as an unresolved doublet with trimethylsilylcodeine and could easily escape detection in a flame ionization detector or total ion-current recording. Figure 3 shows the ion-current plots for the ion pairs 340/343, 414/417, and 430/433. The occurrence of peaks in the $m/e$ 340, 414, and 430 plots coincident with the internal standard peaks in the $m/e$ 343, 417, and 433 plots provides conclusive evidence for morphine in the urine. In spite of the structural similarity of trimethylsilylcodeine and bis(trimethylsilyl)morphine, the former only contributes to the ion current at $m/e$ 343. Based on the 414/417 and 430/433 ion-current
ratios, the concentration of morphine in the urine was calculated to be 77 ng/ml. It seemed likely in this case that the morphine was solely a metabolic product of the ingested codeine. Although morphine is a known metabolite of codeine (16), this fact may not be as widely recognized as it should be in view of the possibility that its detection in urine could be falsely incriminating.

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References
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