curonidase (from Patella vulgata, Type L-II, cat. no. G8132) were purchased from Sigma Chemical Co. (St. Louis, MO) and stored in a desiccator at -20°C. We reconstituted 50-150 mg of the lyophilized enzyme with 10 mL of de-ionized water before use. Before the hydrolysis, we added 900 ng of trideuterated morphine and 150-300 ng of morphine glucuronide or sulfate to 3.0 mL of drug-free urine collected from laboratory employees. For enzymatic hydrolysis, we added 7-21 kU of β-glucuronidase and adjusted the pH to 4.0-5.0. For acid hydrolysis, we added 0.5 mL of concentrated HCl and heated the mixture to 120°C for 20 min. Morphine was extracted with Bond-Elut Certify columns (Analytichem International, Harbor City, CA), and the pentfluoropropionyl derivative was generated (2) before quantification by GC/MS.

Recovery of enzymatically hydrolyzed morphine glucuronide was consistently low and did not differ markedly from the amount of free morphine generated in the absence of enzyme, presumably from nonenzymatic hydrolysis. Varying the temperature, pH, or duration of enzymatic hydrolysis did not improve recovery. Acid hydrolysis was only 30-40% effective in releasing free morphine from the glucuronide conjugate. Comparably results were obtained with two different lot numbers of both enzyme and morphine glucuronide, with morphine glucuronide obtained from a different source (Alltech Associates Inc., Deerfield, IL), and with two additional sources of glucuronidase enzyme (Type HP-2, from Helix pomatia, cat. no. G7017, Sigma Chemical Co., and β-glucuronidase/aryl sulfatase from H. pomatia, cat. no. 127696, Boehringer Mannheim Biochemicals, Indianapolis, IN). Aliquots of three CAP Forensic Urine Drug Testing Survey samples containing morphine were hydrolyzed with acid or enzyme; the quantitative results are compared in Figure 1. β-Glucuronidase hydrolyzed the conjugated morphine in UDC-37 and UDC-09; UDC-08 apparently contained only nonconjugated morphine. When morphine glucuronide was added to survey specimen UDC-09, however, the exogenous conjugate was not hydrolyzed.

The reason for the absence of hydrolytic activity toward the exogenous substrate is not clear. Morphine can be conjugated at either of two hydroxyl groups, although conjugation at the phenolic hydroxyl group to produce the inactive morphine-3-β-D-glucuronide metabolite is highly favored by liver glucuronoyl transferase enzymes. Morphine-6-β-D-glucuronide is a minor metabolite, and the possibility exists that the enzyme may be active toward the 6- but not the 3-conjugate, but this has not been confirmed. Moreover, it seems unlikely that the survey specimens would contain 6-conjugate rather than the physiologically relevant 3-conjugate. Structural impurity of the exogenous morphine-3-β-D-glucuronide would also explain these results, but this compound is insufficiently volatile for conventional analysis by GC/MS. In addition, urine containing added conjugate is positive for opiates by enzyme-multiplied immunoassay (EMIT; Syva Co., Palo Alto, CA). We are continuing to investigate this curious finding in our laboratory.

References

Quantification of CA 125 in Peritoneal Fluid: Inhibition of the CA 125 Response in the One-Step Immunoradiometric Assay and Establishment of a Two-Step Assay Format, Roy F. P. M. Kruijswagen,1 Rob J. van den Berg,1 Martin F. G. Segers,1 Lambert G. Poels,2 Wim N. P. Willemse,1 Rune Rolland,2 and Chris M. G. Thomas1,2 (1 Dept. of Obstet. & Gynecol., Sint Radboud Hosp., and 2 Dept. of Cell Biology and Histology, Catholic Univ., P.O. Box 9101, 6500 HB Nijmegen, The Netherlands; author for correspondence)

We have tested the accuracy of the one-step assay format [i.e., 100 µL of sample incubated for 20 h at room temperature (RT) along with capture- and signal-monoclonal antibody (MAb)] vs the two-step procedure [i.e., 100 µL of sample incubated first (20 h, RT) with capture-MAb, then with signal-MAb (3 h, RT)] for quantifying CA 125 in peritoneal fluid (PF) by immunoradiometric assay (IRMA).

Klug et al. (1) reported that the most practical procedure for assaying CA 125 in serum samples is the one-step RMA, although our experiments with either assay format demonstrate excellent parallelism between serially diluted serum samples over a wide range of sample volumes (0.1-3.12 µL) and the respective standard dose–response curves (range: 7.5-500 arb. units/mL), as shown in Figure 1. Sample concentrations are calculated by correcting the concentrations of the unknowns as read off the standard curve according to the dilution factor (i.e., 100 µL/µL of unknown).

Several groups (2, 3) applied the one-step assay format to measure CA 125 in PF from women with various stages of endometriosis. They reported PF concentrations ranging between 11 and 375 arb. units/mL, but these results appear to be inaccurate because the PF dilution curves did not parallel the standard dose–response curves (2). Using either assay format, we observed that serially diluted PF

Fig. 1. Measured morphine concentration in three CAP Survey urine specimens

UDC-37 was from 1989 Set D, UDC-08 and 09 were from 1990 Set A. Survey means: ---. The hydrolysis experiment was repeated after addition of morphine sulfate (MS) or glucuronide (MG), 500 µg/L, to UDC-09. Specimen (3 mL) were hydrolyzed either enzymatically with 7 kU of β-glucuronidase for 3 h at 80°C or with 0.5 mL of concentrated HCl at 120°C for 20 min.* concentration <10 µg/L.
samples (three from cases of endometriosis, two from women with a normal-looking pelvis) show a high-dose "hook" effect in the one-step IRMA (Figure 1, upper panel), but excellent parallelism in the two-step format (Figure 1, lower panel).

To explain the different behavior of PF in the one-step IRMA, we speculate that the structure of the CA 125 molecule in PF differs from that in serum. This analyte in PF may demonstrate a much lower affinity for the capture MAb (solid phase–liquid interaction) than for the same signal MAb (liquid–liquid interaction). The concentrations obtained from the dilution series of the five PF samples (two-step format) compared with the results from the 50-μL PF samples (one-step format; these results in parentheses) are 1800 (440); 2400 (140); 4800 (200); 5600 (380); and 6700 (150) arb. units/mL, respectively.

In conclusion, (a) the previously reported concentrations of CA 125 in PF determined with the one-step IRMA are not accurate; (b) the limited parallelism of nonserum unknowns in the one-step IRMA can be checked by assaying at least two different sample volumes of PF to ensure parallelism; (c) to avoid nonparallelism in these cases, kit manufacturers should include a two-step assay procedure with their kit information; (d) the much higher CA 125 concentrations in PF presented here may have implications for current pathophysiological theories regarding increased concentrations of CA 125 in serum and PF of women with endometriosis.

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References