New Electrochemiluminescent Immunoassay for the Determination of CYFRA 21-1: Analytical Evaluation and Clinical Diagnostic Performance in Urine Samples of Patients with Bladder Cancer

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Background: A new electrochemiluminescent immunoassay (ECLIA) has been developed for the determination of cytokeratin 19 (CYFRA 21-1) in the Elecsys 2010 immunoassay system. Urinary CYFRA 21-1 might have a role in the diagnosis of bladder cancer.

Methods: We performed an analytical evaluation of the CYFRA 21-1 ECLIA for serum and urine samples. The clinical value of urinary CYFRA 21-1 for the detection of bladder cancer was evaluated through its measurement in 226 urine samples from symptomatic and asymptomatic controls.

Results: At concentrations of 2–30 μg/L, within-assay imprecision (CV) was below 2.1% for sera and 3.3% for urines, with interassay CVs below 3.3% for sera and 4.9% for urines. The day-to-day CV was <20% at concentrations >0.2 μg/L (functional sensitivity). Measurement of diluted samples showed that the assay estimated CYFRA 21-1 between 98% and 103% for sera and 98% and 105% for urines. Recovery of added CYFRA 21-1 was 99–105% for sera and 96–115% for urines. We separately compared serum and urine CYFRA 21-1 ECLIA results with those obtained with an IRMA (CIS bio international). Regression analysis for sera was: CYFRA 21-1 (ECLIA) = 0.520 + 1.018 CYFRA 21-1 (IRMA); [95% confidence interval (CI) (y-intercept), −0.260 to 1.309]; 95% CI (slope), 0.978–1.060; n = 100; $S_{p/y} = 3.242; r^2 = 0.987$. For urine samples it was: CYFRA 21-1 (ECLIA) = 0.716 + 0.966 CYFRA 21-1 (IRMA); 95% CI (y-intercept), 0.009–1.422; 95% CI (slope), 0.956–0.976; n = 100; $S_{p/y} = 4.136; r^2 = 0.986$. In urine samples voided by patients with and without bladder cancer, the best ROC analysis discrimination provided 81.0% (95% CI, 72.7–87.7%) sensitivity and 97.2% (95% CI, 90.2–99.6%) specificity at a threshold value of 5.7 μg/L.

Conclusions: Our initial evaluation showed reliable analytical performance for urinary CYFRA 21-1, which might assist urologists in the detection of bladder cancer as a noninvasive adjunct to cystoscopy.

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Cytokeratins are intermediate filaments expressed by all epithelial cells and which appear to be useful markers of epithelial differentiation (1). CYFRA 21-1 measures cyto-keratin fragments of cytokeratin 19 with the aid of two specific monoclonal antibodies (mAbs):3 BM 19.21 as the capture mAb and KS 19.1 as the detector mAb. The target sites for the CYFRA 21-1 mAbs lie within amino acids 346–367 for BM 19.21 and within amino acids 311–335 for KS 19.1. Cytokeratin 19 consists of 400 amino acids; thus both epitopes are located in the C-terminal helical region of the molecule (2). Serum CYFRA 21-1 has been used as a tumor marker for the diagnosis of malignancies of different origin (3, 4), but most of all in non-small-cell lung cancer (5, 6). It has even been used as a prognostic and diagnostic marker for transitional cell carcinoma (TCC) of the bladder (7), showing low sensitivities and specificities in serum, a fact that suggests a search of different samples to increase these diagnostic characteristics. More than 20 different human cytokeratins have been described, and a given epithelium can be characterized by a specific pattern of cytokeratin expression (8). This

Footnotes:

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Received November 19, 1998; accepted July 12, 1999.

3 Nonstandard abbreviations: mAb, monoclonal antibody; TCC, transitional cell carcinoma; NMP22, nuclear matrix protein-22; ECLIA, electrochemiluminescent immunoassay; IB, Boehringer Mannheim; and 95% CI, 95% confidence interval.
known characterization of cytokeratins in both healthy urothelium and urothelial tumors suggested that the presence of these filaments or their fragments in urine, which is in direct contact with the tumor, could be used as a potential tumor marker for TCC.

Bladder cancer is among the 10 most frequent cancers worldwide. Diagnostic procedures in patients with bladder cancer symptoms include urine cytology, cystoscopy with biopsy, and excretory urography, but cystoscopy remains the reference method to detect primary or recurrent TCC (9). The depth of muscle invasion is used to classify the clinical stage, and differentiation and histological characteristics are used to grade the tumor. Treatment and prognosis depend on these latter features (10). Superficial tumors are transurethral resected and/or receive intravesical chemotherapy (11), invasive tumors are deep resected and treated with systemic therapy, or cystostomized (12), and metastatic tumors are treated with systemic chemotherapy. More than 50% of the superficial tumors recur within 5 years, and 10–20% of these progress into invasive disease. Prognosis is multifactorial, and a regular follow-up is required (13). There is a need for objective noninvasive methods that could help the urologists in the diagnosis of the disease. Cytopathology and flow cytometry as diagnostic tools have attempted to replace cystoscopy, but their sensitivities are not high enough in well or moderately differentiated tumors (14). Antigens such as M344 have been described for this purpose (15). Alternative urinary biomarkers such as Bard tumor antigen (16), nuclear matrix protein-22 (NMP22) (17), and fibrin/fibrinogen degradation products (18) have recently been approved by the Food and Drug Administration for clinical use for detecting recurrence of disease. Many others, including urinary cytokeratins such as the new urinary bladder cancer antigen (19) have been developed. They all continue to be evaluated as potential tools that might guide the urologist as to the need for cystoscopy and might increase the interval of endoscopic evaluations.

We report our analytical evaluation of the CYFRA 21-1 assay according to ECCLS guidelines (20). In this study we evaluated the functional sensitivity, intra- and interassay precision, linearity, recovery, and carryover for serum and urine samples. The electrochemiluminescent immunoassay (ECLIA) method was compared with a manual IRMA (CIS bio international). Once we had demonstrated that the test worked analytically in both samples, we then performed a clinical evaluation. We studied the diagnostic performance of the CYFRA 21-1 assay in the urines of patients with bladder cancer and not in serum, where previous studies have shown that it is not efficient enough for the diagnosis of the disease (7).

**Materials and Methods**

**ELECSYS 2010 SYSTEM**

The Elecsys® 2010 analyzer [Boehringer Mannheim (BM)] is based on the ability of the electrochemiluminescent label molecule, a tris(2,2’-bipyridyl)ruthenium (II) complex, to be repeatedly excited by tripropylamine, thus leading to an amplification of light signal that allows the high speed and dynamics of signal generation and measurement. It provides the first test result in 18 min and has a maximum throughput of 86 tests per hour. The system can develop both competitive and sandwich-format electrochemiluminescent assays.

The Elecsys 2010 system is a fully automated immunoassay analyzer that can work in batch, random, or stat modes. The automated process consists of the aspiration of the sample, reagent and microparticles, a first incubation at 37 °C, additional reagent pipetting, a second incubation at 37 °C, reaction mixture aspiration, and measurement. The analyzer also includes a workstation for system programming and can be interfaced to various laboratory computers.

**ELECSYS 2010 CYFRA 21-1 ASSAY**

No preanalytical preparation of reagents is required for the Elecsys 2101 CYFRA 21-1 assay (cat. no. 1820966). In a first incubation of 9 min, 20 μL of sample, a biotinylated monoclonal cytokeratin 19-specific antibody, and a monoclonal cytokeratin 19-specific antibody labeled with a ruthenium complex [a tris(2,2’-bipyridyl)ruthenium (II) complex] react to form a sandwich complex. After the addition of streptavidin-coated microparticles, there is a second incubation for 9 min, and the complex becomes bound to the solid phase via the interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with a phosphate-tripropylamine buffer (pH 6.8; Procell®, BM). Application of a voltage to the electrode then induces chemiluminescent emission, which is measured by a photomultiplier.

**COMPARISON METHOD: CYFRA 21-1 IRMA**

An IRMA (CIS bio international) based on the sandwich principle was used for the method comparison study for CYFRA 21-1. Calibrators, controls, and samples were added to anti-CYFRA 21-1 antibody-coated beads and a second anti-CYFRA 21-1 antibody labeled with 125I. The excess of labeled antibody was washed, and the sandwich was detected using a gamma counter.

**CALIBRATION**

We calibrated the ECLIA CYFRA 21-1 assay with two calibrators (Elecsys 2010) at two different concentrations, 5 and 50 μg/L of analyte. Every instrument-specific calibration curve is generated by a two-point calibration and a master curve provided via the reagent barcode. IRMA CYFRA 21-1 concentrations were calculated from a calibration curve determined by the concurrent testing of calibrators in each analysis.
CONTROLS
We used PreciControl Tumor Marker controls (TM1 and TM2) from BM (cat. no. 1776452) at two different concentration ranges: TM1 (3.7–8.2 µg/L) and TM2 (23.2–37.0 µg/L). All of these controls for CYFRA 21-1 were human serum based. These controls covered the lower analytical range of the assay, where more precision is needed for clinical purposes. We also used pooled human sera and pooled urines at different concentrations for the imprecision study.

HUMAN SERA AND URINES
Sera and urines from 224 subjects including healthy subjects and patients who required laboratory testing were used for the precision, linearity, recovery, carryover, and method comparison studies and the clinical diagnostic validation. Urines were centrifuged at 1000 g for 10 min at 4 °C and stored at −80 °C until processing.

ANALYTICAL EVALUATION
Functional sensitivity of CYFRA 21-1. Sera and urines from patients were mixed with a serum or urine, respectively, of a known CYFRA 21-1 concentration to obtain serum or urine pooled samples with of CYFRA 21-1 concentrations of 0.1–2.0 µg/L. These pools were aliquoted, frozen at −80 °C, and tested on 10 different nonconsecutive days during 1 month. Three different calibration lot numbers and two software versions of the Elecsys 2010 system were used during this study. The functional sensitivity was obtained directly from curve fitting of the mean concentration of every sample and its respective CV over the 10 measurements after a quadratic regression fit method. Functional sensitivity was defined as the lowest concentration of analyte measured with an interassay CV of ≤20% (21).

Precision studies. The precision of the Elecsys 2010 CYFRA 21-1 was evaluated according to NCCLS protocol EP5-T2 (22). The two BM test point ligand controls and different human serum and urine pools at clinically important analyte concentrations were analyzed 10 times a day for the intraassay imprecision study and on 10 nonconsecutive days during 1 month for the interassay imprecision study.

Linearity studies. Three serum samples with different concentrations of CYFRA 21-1 were diluted with a protein matrix diluent (Universal® BM diluent; cat. no. 1732277) at 1:2 (100 µL sample + 100 µL diluent), 1:5 (100 µL sample + 400 µL diluent), and 1:10 (100 µL sample + 900 µL diluent) for serum and urine. The evaluations were made by the percentage of difference between the expected and the observed values.

Recovery studies. Different amounts of a serum or urine containing a high concentration of CYFRA 21-1 were added to serum and urine samples at different concentra-

CLINICAL EVALUATION
Subjects. Four groups of subjects entered the study. Group 1 included 86 patients with an active TCC as confirmed by their positive cystoscopies and who were scheduled to have transurethral resections or cystectomies. Group 2 consisted of 76 follow-up patients with a history of TCC who were free from disease as confirmed by negative cystoscopies at the time of the study. Group 3 included 32 patients with other urological pathological conditions (including 2 with kidney carcinoma, 3 with neurogenic bladder, 8 with urethral lithiasis, 9 with prostatic carcinoma, and 10 with urinary tract infection). Group 4 consisted of 30 healthy subjects free of urological diseases. These volunteers, whose self-reported current health status was not confirmed by medical examination, were recruited from the staff of the hospital. Subjects with a previous diagnosis of malignancy other than bladder cancer of any histology (paying attention to lung cancer) or TCC of the renal pelvis, ureters, or urethra (except nonmelomato skin cancer) were excluded from the study.

Carryover studies. The Broughton carryover percentage (K) was calculated according to the equation: carryover (%) = \[\frac{(L_1 - L_3)/(H_3 - L_3)}{L_1}\] × 100 (23). K was estimated in serum and urine samples to assess carryover. Three consecutive sera and urines with high (H) CYFRA 21-1 concentrations were measured, followed by three with low (L) CYFRA 21-1 concentrations, and this sequence was repeated five times.

Method comparison studies. Serum and urine samples from 195 subjects were collected for the method comparison study; serum and urine results were separated. Patients with bladder cancer, others with benign and malignant pathologies, and healthy individuals were included to cover the CYFRA 21-1 analytical range, with careful attention paid to clinically important concentrations. Most of the samples included in the method comparison evaluation were the same as those included in the clinical study. There were five patients with bladder cancer for whom CYFRA 21-1 was measured in urine and serum samples. These patients had superficial tumors, and serum concentrations were not increased over the reference interval, according to the low sensitivity of the test in serum samples for the detection of bladder cancer. The Elecsys 2010 CYFRA 21-1 assay was compared with a manual IRMA (CIS bio international). The statistical evaluations were made by linear regression. We have included a regression plot for CYFRA21-1 values below 10 µg/L in both serum and urine evaluations because the majority of the samples for the regression study were within this range.
Histopathological data were also recorded. Cases of group 1 who received surgical treatment were stratified by extent of disease as superficial (pTa, pT1) or muscle invasive (pT2, pT3, pT4) and by tumor grading (I, II, and III), according to the American Joint Committee on Cancer TNM criteria. Only the five major types of bladder cancer were taken into account for purposes of homogeneity (two cases of carcinoma in situ were excluded).

All patients participating in the study were informed and gave their consent according to the procedures approved by the ethics committee of our institution.

**Samples.** A total of 224 subjects provided urine voided samples for analysis. Preoperative urines from group 1 were collected in the operating theater immediately before or within 6 weeks before the surgical procedure. Voided samples from patients of group 2 were collected before the performance of the cystoscopies, which qualified them for inclusion in this group. No patient in groups 1 or 2 received chemotherapy or immunotherapy during the time of sample collection. To provide the diversity expected in clinical practice involving patients with these benign or malignant urological diseases, samples from group 3 were recruited consecutively during a period of 2 weeks. Samples from group 4 were collected when the subjects were not under a physician’s care for any condition or disease of the genitourinary tract and had no symptoms suggesting that such a condition was present. In addition, these urines were submitted to urinalysis to exclude urinary tract infections.

**Statistical Analysis**

Data were reported as the mean [including SD, 95% confidence intervals (95% CI), and standard error of the mean (SE)], median, and range. Statistical inferences were evaluated using nonparametric tests. Differences between two means were evaluated using the Mann–Whitney U-test. Differences among three or more groups were evaluated using the Kruskal-Wallis nonparametric one-way ANOVA. A P value of 0.05 was considered statistically significant. Sensitivities, specificities, and ROC curves were also determined to obtain the cutoff that offered the best sensitivity and specificity (including the 95% CI) combination defined by the largest area under the curve.

**Urinalysis**

Urinary leukocytes, nitrites, and hemoglobin were measured on all subjects entering this study to exclude urinary tract infections and hematuria as possible interferences in the clinical interpretation of the test. Urinalysis was performed by colorimetric methods using Combur-Test M strips, cat. no. 1379208 (BM), which were read in a Miditron® photometer.

**Results**

**Analytical Evaluation**

**Functional sensitivity for CYFRA 21-1.** The functional sensitivity for CYFRA 21-1 (CV = 20%) was 0.2 μg/L (Fig. 1).

**Precision studies.** The statistical analyses of the precision studies are shown in Table 1. The within-run CVs were 1.1–2.1% for serum samples and 1.8–3.3% for urine samples. The between-day CVs varied from 2.8% to 3.3% for sera and from 2.2% to 4.9% for urines.

**Linearity and recovery.** The results of the dilution linearity and recovery studies are shown in Table 2.

**Carryover.** Carryover percentages expressed as K described by Broughton (23) were K = 0.003% for serum samples and K = 0.004% for urine samples.

**Method comparison studies.** The relationship between the CYFRA 21-1 ECLIA and the CYFRA 21-1 IRMA is shown in Figs. 2 and 3. For the serum samples, in which the CYFRA 21-1 concentration ranged from 0.5 to 100 μg/L, the 95% CI of the slope was not statistically different from 1.0 and the 95% CI of the intercept was not statistically different from zero. For serum CYFRA 21-1 values below 10 μg/L, the 95% CI of the slope also was not statistically different from zero.

**Table 1. Intra- and Interassay Imprecision for the Elecsys CYFRA 21-1 assay.**

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean, μg/L</td>
<td>CV, %</td>
</tr>
<tr>
<td>Intraassay precision</td>
<td>2.5</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>6.7 (TM1)</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>31.2 (TM2)</td>
<td>1.1</td>
</tr>
<tr>
<td>Interassay precision</td>
<td>2.6</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>6.7 (TM1)</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>31.2 (TM2)</td>
<td>3.2</td>
</tr>
</tbody>
</table>
different from 1.0, whereas the 95% CI of the intercept was statistically different from zero. For the urine samples, in which the CYFRA 21-1 concentration ranged from 0.5 to 600 μg/L, the 95% CI of the slope was statistically different from 1.0 and the 95% CI of the intercept was statistically different from zero. For urine CYFRA 21-1 values below 10 μg/L, the 95% CI of the slope remained statistically different from 1.0 and the 95% CI of the intercept remained statistically different from zero.

### Clinical Evaluation

Demographic data included age and sex, and the distribution for each group is shown in Table 3. Patients with TCC were older than patients in group 3 and the healthy volunteers (P < 0.05). The male-female ratio was higher in the patient groups than in the control group (P < 0.05).

The CYFRA 21-1 results for all groups are summarized in Table 4. The mean CYFRA 21-1 concentration in group 1 was 125.7 μg/L, which was significantly higher than those of patients in group 2 (3.3 μg/L; P < 0.001), patients in group 3 (9.6 μg/L; P < 0.001), and healthy controls (2.7 μg/L; P < 0.001). All combinations among groups 2, 3, and 4 were also significant, even between groups 2 and 4 (P = 0.029). The descriptive statistical parameters reflecting the distribution of urinary values in the four groups is shown in Table 4.

Histopathological staging of group 1 included 22 patients with stage pTa tumors, 41 with stage pT1, 7 with stage pT2, 9 with stage pT3, and 3 with stage pT4. There were 30 patients with a grade I tumors, 26 with grade II tumors, and 26 with grade III tumors. Of the 61 patients of group 1 with superficial tumors, 30 had grade I, 24 had grade II, and 9 had grade III tumors. For the invasive tumors, no patients had grade I, 2 had grade II, and 17 had grade III tumors. No statistical difference of CYFRA21-1 among stages (P = 0.340) was found (Table 5). The Mann–Whitney U-test showed significant differences only between pTa and pT1 stages (P = 0.044). Within group 1, patients with superficial bladder cancer (pTa, pT1) had a mean urinary CYFRA 21-1 concentration of 131.0 μg/L, whereas in invasive tumors (pT2, pT3, and pT4), the mean CYFRA 21-1 concentration was 122.0 μg/L. These concentrations were significantly different, and both were significantly higher than those of groups 3 and 4.

The Kruskal–Wallis test indicated significant differences among grades (P = 0.028; Table 5). When differences between consecutive grades were analyzed statistically through the Mann–Whitney U-test, grades I and II were different (P = 0.019) but not grades II and III (P = 0.572).
The diagnostic profile of CYFRA 21-1 was evaluated through ROC analysis (Fig. 4). The optimal combination of 81.0% (range, 72.7–87.7%) sensitivity and 97.2% (range, 90.2–99.6%) specificity was obtained from the ROC analysis using a threshold value of 5.7 mg/L. When we defined a specificity of 95%, which was one of the most convenient, the ROC analysis gave a cutoff of 5.4 mg/L, which increased the sensitivity to 81.9% (range, 73.7–88.4%).

Two-thirds of the urines demonstrated microhematuria and one-fifth showed gross hematuria. We could assess the differences in CYFRA 21-1 values among hematuric and nonhematuric urine voided samples in a group of five patients who had intermittent periods of hematuria during the study (data not shown). These differences were in all cases <5%. The effect of urinary tract infections as a possible interference in the clinical interpretation of the test was evaluated as well, through the urinary dipstick test. When a urine was suggestive as positive for a urinary tract infection, the sample was excluded from the study and a new urine was required.

### Table 3. Characteristics of subjects entering the study.

<table>
<thead>
<tr>
<th>Group 1 (Active bladder cancer)</th>
<th>Group 2 (No evidence of residual disease)</th>
<th>Group 3 (Other urological diseases)</th>
<th>Group 4 (Healthy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>86</td>
<td>76</td>
<td>32</td>
</tr>
<tr>
<td>Sex distribution, M/F</td>
<td>67/19</td>
<td>62/14</td>
<td>48/14</td>
</tr>
<tr>
<td>Mean age (SE), years</td>
<td>66.9 (1.2)</td>
<td>76.6 (0.7)</td>
<td>56.5 (3.3)</td>
</tr>
</tbody>
</table>

### Table 5. CYFRA 21-1 concentrations (µg/L) relative to histological data of patients from group 1.

<table>
<thead>
<tr>
<th>Stage&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean</th>
<th>SD</th>
<th>SE</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTa</td>
<td>136.2</td>
<td>330.5</td>
<td>75.8</td>
<td>24.0</td>
<td>2.2–1387</td>
</tr>
<tr>
<td>pT1</td>
<td>133.1</td>
<td>280.2</td>
<td>43.8</td>
<td>51.6</td>
<td>3.0–1658</td>
</tr>
<tr>
<td>pT2</td>
<td>170.7</td>
<td>211.1</td>
<td>79.8</td>
<td>133.9</td>
<td>2.8–590</td>
</tr>
<tr>
<td>pT3</td>
<td>105.8</td>
<td>109.8</td>
<td>36.6</td>
<td>44.3</td>
<td>11.4–291</td>
</tr>
<tr>
<td>pT4</td>
<td>95.1</td>
<td>86.8</td>
<td>50.1</td>
<td>93.6</td>
<td>9.0–182.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Grade&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mean</th>
<th>SD</th>
<th>SE</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>104.1</td>
<td>274.5</td>
<td>51.9</td>
<td>22.3</td>
<td>2.3–1387</td>
</tr>
<tr>
<td>II</td>
<td>142.9</td>
<td>327.7</td>
<td>65.5</td>
<td>56.0</td>
<td>2.8–1658</td>
</tr>
<tr>
<td>III</td>
<td>153.6</td>
<td>36.3</td>
<td>18.5</td>
<td>76.0</td>
<td>3.8–600</td>
</tr>
</tbody>
</table>

<sup>a</sup>, <sup>b</sup> Kruskal-Wallis one-way ANOVA: <sup>a</sup> p = 0.340; <sup>b</sup> p = 0.028.

The minimum detectable concentration for the ECLIA CYFRA 21-1 could not be estimated without information from the master calibration curve provided by the barcode card (BM). The lower detection limit described by the manufacturer was better than those of other commercial manual enzymoimmunosorbent assays carried out in fully automated analytical systems (24, 25).

We found it more interesting to evaluate the functional sensitivity (21), which is preferable for the evaluation of all low-range tumor marker concentrations of clinical relevance, either at initial diagnosis or at follow-up. Our functional sensitivity study was performed in the actual conditions of a routine clinical laboratory: we measured the sera and urine pools using three different calibrator lots and used two different software versions and two different automated systems. Inconsistencies in functional sensitivities can be found among laboratories using the same automated system. These discrepancies can be attributed to the way these sera and urine pools are prepared, the periodicity of measuring, and even to differ-
ences among automated systems that theoretically are the same. It is important that the measurements of low CYFRA 21-1 concentrations are reliable, but at present most CYFRA 21-1 semi- or fully automated systems provide good sensitivities that allow differentiation between tumoral increments (25).

The within-run and between-day CVs for both serum and urine samples were <5%, as recommended for tumor markers. The reproducibility of this CYFRA 21-1 assay could be considered as acceptable, especially because the urine samples had been frozen at −80 °C, which might have implied instability. We have not found differences in stability between urine CYFRA 21-1 stored at −20 °C and at −80 °C (data not shown). The precision performance of the Elecsys 2010 system for this assay was better than the performance reported for some manual, semi-automated, or fully automated assays (24, 25).

The dilution studies performed for the Elecsys 2010 CYFRA 21-1 assay with the respective BM diluents were quite acceptable. The percentages of recovery showed a worse profile in urine samples than in sera. No matrix effect was apparent with the manufacturer’s recommended diluent.

The carryover study showed good percentages in both samples. This observation is to be expected when working with an automated system that changes its tips and cuvettes with every sample.

Generally, hemolysis, lipemia, and icterus are less significant for immunoassays or competitive binding assays than for the classic colorimetric analytical methods. We did not evaluate these specimen-based interferences, which usually have a method-specific nature, because they are less usual in sandwich methods such as the ECLIA CYFRA 21-1 assay (26). Nevertheless, many different categories of serum and urine samples were tested in this study. For this analyte, no significant difference was observed between the Elecsys 2010 and the comparative assay in patient serum samples with increased bilirubin, rheumatoid factor, immunoglobulins, or triglycerides, or in the presence of blood cells in both sera and urines.

According to the manufacturer’s guidelines, there is no high-dose hook effect for CYFRA 21-1 concentrations up to 2000 μg/L. This possibility of falsely low concentrations of CYFRA 21-1 should be less frequent in this ECLIA method than in an IRMA assay because of the wider measuring (reportable) range of the Elecsys method (0.1–500 μg/L) compared with the IRMA (0.5–60 μg/L).

We also tested the specificity of the CYFRA 21-1 assay by measuring calibrators of high concentrations of tissue polypeptide antigen (cytokeratins 8, 18, and 19) (2, 7) with a routine method applied in our laboratory to measure this tumor marker (IRMA; Sangtec Medical; data not shown). We did not find any cross-reactivity in the Elecsys CYFRA 21-1 assay, but a small matrix effect appeared that was related to the utilization of calibrators prepared for different purposes and techniques. Our observation is in accordance with other studies evaluating different methodologies and automated systems for CYFRA 21-1 (27).

The global and partial regression analysis of this correlation study between ECLIA and IRMA assays for CYFRA 21-1 gave slopes that were not statistically different from 1.0 for serum samples, a finding that might suggest no differences in assay calibration. Slightly greater differences were found for urine samples, which might be attributed to the differential characteristics of this latter sample and the differences associated with the repeated need for dilution when processing urine samples with the IRMA because of its reduced measurement range (0.5–60 μg/L). There seemed to be a proportional or constant error in the regression studies for both the serum and urine analyses, even more when values below 10 μg/L were taken into account, an observation that could...
be attributed to the different lower limits of detection for both techniques (0.1 µg/L for ECLIA and 0.5 µg/L for IRMA) and the fact that the ECLIA is fully automated and the IRMA is manual.

The development of bladder cancer includes an early step related to cellular damage, which could be followed by cellular differentiation and exophytic growth. During this process, bladder cells might be expected to be exfoliated into urine, and all of their intracellular components could be detected within urine voided samples. Cytokeratins as intracellular filaments are supposed to be present in different concentrations in the urine of patients with bladder cancer, depending on their proliferation and production rates and the different degrees of exfoliation that could appear in the different subtypes of bladder cancer.

One of the first steps in the evaluation of a tumor marker is the establishment of the reference interval. CYFRA 21-1 had a mean concentration of 2.7 µg/L in the healthy subjects, which is slightly higher but consistent with that of 2.3 µg/L reported in a group of 36 subjects free of urothelial disease in a recent study using an ELISA methodology (28). The mean concentration in the healthy group was apparently similar to that of the patients with a previous bladder cancer but free of disease, but some statistical difference was found (P = 0.029). The higher CYFRA 21-1 concentration in group 2 might be attributed to a previous bladder cancer with residual tumor or malignant cells remaining because of field disease, but not enough to reach the cutoff that differentiates the active processes from individuals free of disease. Patients with urological diseases other than bladder cancer had higher CYFRA 21-1 concentrations than groups 2 and 4, possibly because of exfoliation of bladder cells in pathologies such as neurogenic bladder, lithiasis, or infections of the urinary tract. In patients with prostatic and kidney carcinomas, CYFRA 21-1 concentrations were higher than in patients with benign pathologies (group 3), a fact that could be explained by an increased production of keratins by these malignancies with a pattern of epitopes similar to those in bladder tumors that also react with the anti-CYFRA 21-1 antibodies of the assay and form the sandwich detectable by ECLIA. This observation might suggest that the increased production of keratins in malignancies of origins other than the bladder could be found in the urine via kidney filtration. This might reflect a lack of specificity of cytokeratins as urinary tumor markers as has already been found in serum (3–7). The statistical difference among the active bladder cancer group and the remaining groups indicated that CYFRA 21-1 might differentiate bladder tumors from the rest of subjects tested for diagnosis purposes.

Regarding stage, only patients with superficial diseases pTa and pT1 had significant differences in their CYFRA 21-1 concentrations (P = 0.044). Among invasive diseases, no statistical differences were found. CYFRA 21-1 appeared to be differential mainly in superficial rather than in invasive tumors. Superficial and invasive diseases are known to be pathophysiologically different enough to lead to a different treatment and prognosis. In invasive tumors, CYFRA 21-1 concentrations were lower than in superficial tumors. One explanation could be a change in the cell exfoliation between superficial and invasive stages, where cancer growth is inward. A second could be related to a differential mechanism of cytokeratin release of the invasive tumors, which might be directly mainly into the blood (2) and not into the urine. This could justify their lower than expected urinary concentrations and the comparative increases of blood concentrations in invasive tumors (7, 24, 28).

In relation to grade, we found a global statistical difference of CYFRA 21-1. The absence of differences between patients with grades II and III could be attributed to the well-known difficulties in defining a tumor as moderately differentiated (grade II) or undifferentiated (grade III), which has long been recognized by pathologists (29).

We investigated whether CYFRA 21-1 could be relevant in the diagnosis of bladder cancer through the ECLIA determination of fragments of cytokeratins in urine voided samples. Specificity for the diagnostic evaluation of CYFRA 21-1 by ROC analysis was obtained through values in patients from group 2, who were free from disease at the moment of study as confirmed by cystoscopy, the remaining gold standard diagnostic method for TCC. Sensitivity was obtained from the patients of group 1, whose samples were collected either in the operating theater or in the 2 months before the transurethral resection or cystectomy. This mixed collection of samples for group 1 allowed us to better evaluate the borderline values around the ROC analysis cutoff. Preoperative CYFRA 21-1 concentrations were higher than those urines collected in the previous weeks before the surgical procedure, a fact that also provided confidence in the assay.

The area under the entire ROC curve was 0.930. The optimal threshold point given by ROC analysis was 5.7 µg/L. This evaluation gave a sensitivity of 81.0% and a specificity of 97.2%. If CYFRA 21-1 is to be used as a diagnostic test, it is desirable to maximize sensitivity; therefore, when the specificity was lowered to 95%, predetermined as an optimal figure, the CYFRA 21-1 threshold decreased to 5.4 µg/L and sensitivity increased slightly to 81.9%. The selection of one of these cutoffs is greatly dependent on the most relevant characteristic in each case: a higher specificity or a higher sensitivity.

Intra- and interindividual variations of urine concentrations might justify the establishment of ratios of urinary CYFRA 21-1 to urinary creatinine. We have performed this clinical evaluation without normalization of CYFRA 21-1 within the context of this transversal study, in which all urines were taken just once. To equally balance the different urinary excretions, the need for
normalization appears to be essential in follow-up designs including successively collected urines.

Several clinical conditions such as hematuria and urinary tract infections, both common in patients with bladder cancer, might confound test interpretation (28). Because of the small differences found in those patients with intermittent periods of hematuria, we considered both samples, hematuric or nonhematuric, acceptable for the determination of CYFRA 21-1. We found it more interesting to assess the absence of a urinary tract infection as a possible interference in the CYFRA 21-1 measurement. Patients with asymptomatic urinary infections were shown to confound test interpretation, and samples that were positive for leukocytes and nitrates were excluded from study and a new voided urine was required.

We could not perform urinary cytology on all of the urine samples of the patients in our study. Urinary cytology is known to be insensitive in cases of low-grade papillary and smooth lesions, and sensitivities of ~40% have been described for well-differentiated tumors (14). This fact can be explained in part by the difficulties of preserving and observing low-grade malignant cells in urine; in part by the subjective accuracy of cytology (differences among cytopathologists grading the same bladder tumors could be found); and in part by changes in the exfoliated cells that could be attributed to intravesical chemotherapy, urinary tract infections, or stone lesions.

When we compared CYFRA 21-1 measured by ECLIA with the diagnostic performance of urine CYFRA 21-1 as a potential tumor marker for bladder cancer as determined by different methodologies such as ELISA (24) or IRMA (28) over the last few years, the sensitivity of ECLIA appeared to be greater than the sensitivities reported by Dittadi et al. (24) and Pariente et al. (28), who included fewer cases in their evaluations. In relation to the bladder antigen test (16) measured by the latex agglutination method, which described a 95.9% specificity estimated in healthy volunteers and nonurological patients, CYFRA 21-1 showed even a higher specificity, which was estimated with follow-up patients free of disease as confirmed by cystoscopy. When comparing the efficiency of the promising NMP22 results described by Soloway et al. (17) and CYFRA 21-1 tests to detect bladder cancer, the diagnostic profile through ROC analysis gave a larger area under the curve for CYFRA 21-1 (0.930) vs NMP22 (0.734) and better sensitivities (~81%) for CYFRA 21-1 vs NMP22 (~75%). However, considering the different study populations, it is not possible to imply that one tumor marker is better than another, and comparative studies including these tumor markers as well as cytology appear to be indicated.

The fully automated Elecsys 2010 assay for CYFRA 21-1 in serum and urine samples has the technical capability required in a routine laboratory. This assay is rapid, giving the first result in 18 min. In addition, the stability of the calibration curve of the Elecsys 2010 is at least 2 months, in contrast to the IRMA, which requires a new calibration curve with each protocol. The CYFRA 21-1 assay offers an extended range, allowing a minimal number of sample dilutions. The Elecsys CYFRA 21-1 assay showed a high degree of reproducibility. Its linearity, recovery, and comparison studies were satisfactory, and we did not find any interference or carryover problems. Our preliminary evaluation of the diagnostic performance of CYFRA 21-1 showed that the test is a promising tool that might have a role in the detection of TCC. Taking into account that other urological lesions of the urinary tract and malignancies of prostate and kidney could increase CYFRA 21-1 concentrations, a threshold value of 5.4 µg/L gave a specificity was 95% and a sensitivity of 81.9%. The ECLIA provided an apparently clear separation of patients with TCC from subjects free of TCC.

In conclusion, this evaluation of the Elecsys CYFRA 21-1 assay demonstrated several performance characteristics that contributed to an increase in its reliability. Our initial clinical results showed that CYFRA 21-1 is a potential noninvasive test that can be performed easily, which might assist urologists in the detection of bladder cancer as an adjunct to cystoscopy.

We thank Boehringer Mannheim, Mannheim, Germany for providing reagents free of charge. We are also grateful to Rocío Quiles and Pilar Casasola for assistance in collecting patients samples.

References