# Rapid Determination of Acetaminophen in Physiological Fluids by Liquid Chromatography Using SDS Mobile Phase and ED Detection

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## Abstract

**Acetaminophen is determined in serum and urine samples by a rapid, sensitive, and precise chromatographic method without any pretreatment step in a C18 column using a pure micellar mobile phase of 0.02M sodium dodecyl sulfate at pH 7. Acetaminophen is eluted in less than 5 min with no interference of the protein band. The use of electrochemical and UV detection is compared. Linearities ($r > 0.999$), as well as intra- and interday precision, are studied in the validation of the method. Limits of detection (LOD) are also calculated to be 0.56, 0.83, and 0.74 ng/mL in micellar solution, serum, and urine using electrochemical detection. The developed micellar liquid chromatographic method is useful for the quantitation of acetaminophen in serum and urine. Recoveries in the biological matrices are in the 98–107% range and results are compared with those obtained using a reference method. Drug excretion (in urine) and serum distribution are studied in several healthy volunteers, and no interference from metabolites is found. The developed procedure can be applied in routine analyses, toxicology, and therapeutic monitoring.**

## Introduction

Acetaminophen (paracetamol or *N*-acetyl-*p*-aminophenol) is a synthetic nonopiate derivative of *p*-aminophenol and a major metabolite of phenacetin, which is associated with analgesic nephropathy. Chemically, it is a basic ($\log k = 9.71$) and hydrophilic ($\log P_{o/w} = 0.47$) drug. Pharmacologically, it produces analgesia and antipyresis and is generally nontoxic at therapeutic doses (0.5–1 g). Following oral administration of 500 mg, peak plasma concentration is attained within 60–120 min. It has a plasma half-life of 1.25–3 h, although this may be prolonged following toxic doses or in patients with liver damage (1). Acetaminophen is metabolized predominantly in the liver and excreted in the urine mainly as the glucuronide and sulfate conjugates. Less than 5% is excreted unchanged as acetaminophen (2).

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Definitive indications for therapeutic monitoring of acetaminophen have not been well defined, and the need for drug concentration determination becomes critical in situations where overdose, abuse, or toxicity is suspected. Recommendations are proposed in the following situations: suspected dose-related drug toxicity, acute overdose, chronic abuse, suspected patient noncompliance, change in liver or renal function, and screening for acetaminophen as a co-ingestant. The therapeutic dose of acetaminophen in serum is 10–20 µg/mL (3).

Determination of acetaminophen in biological samples has been extensively studied using high-performance liquid chromatography (HPLC) with aqueous–organic mobile phases and a UV (4–12) or electrochemical detection (ED) (13,14) mode. Several studies have been conducted to extract proteins from biological fluids and to later analyze the drug in conventional liquid chromatography (15,16). These conventional methods usually make use of previous extraction steps, an internal standard, and mobile phases of methanol or acetonitrile.

Micellar LC (MLC), using mobile phases containing a surfactant concentration above its critical micellar concentration, is an alternative to conventional HPLC. The solubilizing ability of micelles is one of their most important properties and allows direct injection of untreated samples, including biological fluids such as serum and urine. A previous MLC method was developed by Love et al. to analyze acetaminophen in serum using a nonionic surfactant with the aim of predicting the retention behavior of several solutes in chromatographic systems containing Brij-35 (17).

In our laboratory, MLC has proven to be a useful technique in the control of several drugs in biological fluids, such as serum (18,19) and urine (20,21), using sodium dodecyl sulfate (SDS) mixed with an alcohol such as propanol, butanol, or pentanol as the mobile phase and UV, ED, or fluorescence detection.

The scope of this work was to develop a simple, fast, and easy-to-use MLC method for the determination of acetaminophen in biological fluids. Direct injection of the samples simplifies the method, and ED increases the sensitivity and selectivity of the determination in the biological matrix. The

proposed method is useful to determine acetaminophen in monitoring routine analyses. The use of pure micellar mobile phases gives the procedure some attractive advantages such as nontoxicity, nonflammability, biodegradability, and a low cost in comparison with aqueous–organic solvents.

## Experimental

### Instrumentation

The pH of the solutions was measured with a Crison GLP 22 (Barcelona, Spain) equipped with a combined Ag/AgCl/glass electrode. The balance used was a Mettler-Toledo AX105 Delta-Range (Greifensee, Switzerland). The vortex shaker and sonication unit were from Selecta (Barcelona, Spain). The chromatographic system was an Agilent Technologies Series 1100 (Palo Alto, CA) equipped with a quaternary pump, autosampler, and UV–vis and electrochemical detectors. A Digicen 20-R centrifuge (Orto Alresa, Madrid, Spain) was utilized to centrifuge serum samples.

### Chemicals and reagents

Acetaminophen was purchased from Fluka Chemie (Buchs, Switzerland). Distilled, deionized water was used throughout. Sodium acetate, SDS, potassium chloride, sodium dihydrogen phosphate, trifluoroacetic acid, acetonitrile, and methanol were from Merck (Darmstadt, Germany). Ortho phosphoric acid was from Panreac (Barcelona, Spain). Sodium hydroxide was from Riedel-de Haën (Seelze, Germany).

### Chromatographic conditions

A reversed-phase Kromasil C18 column (250- × 4.6-mm, 5-µm particle size) and a precolumn (30 mm) of similar characteristics (Scharlab, Barcelona, Spain) were used. The mobile phase was 0.02M SDS buffered with 0.01M $NaH_2PO_4$ at pH 7. The flowrate and injection volume were 1 mL/min and 20 µL, respectively. Experiments were performed at room temperature. Detection was performed at 250 nm in UV and 500 mV in ED. The retention time for acetaminophen was 4.3 min. Chromatographic signals were acquired and treated with a Hewlett-Packard ChemStation (Rev. A.10.01, Palo Alto, CA), which was also used in the calculation of chromatographic parameters.

### Preparation of solutions

*Mobile phases*

The micellar mobile phase was prepared utilizing SDS, which was buffered with sodium dihydrogen phosphate at pH 7, using 0.1M of sodium hydroxide. For comparison purposes, an aqueous–organic mobile phase was prepared, containing acetonitrile and trifuoroacetic acid using a gradient program mixed with acetate buffer at 1 mL/min for the determination of acetaminophen in urine (11). For serum samples, an acetonitrile–water (55:45, v/v) isocratic method at 0.8 mL/min was used (12).

*Stock solutions*

A solution of 100 µg/mL of acetaminophen was prepared. Several volumes were added to the biological samples (serum and urine) and suitably diluted with the micellar mobile phase to the desired concentration before analysis.

*Biological Samples*

Blood samples were collected using a DB SST Tube (BD Vacutainer Systems, Plymouth, U.K.) and centrifuged for 5 min at 3000 rpm at 4ºC; the serum was stored. Urine samples were collected in a urine collection cup (BD Vacutainer Systems). Analyses were performed with a few milliliters of serum or urine. Biological samples were directly injected into the chromatographic system after 1:10 dilution with 0.02M SDS. Before injection, solutions were filtered directly through 0.45-µm nylon membranes into the autosampler vials.

### Equilibration and care of the column

Use of the same column with the same type of surfactant (anionic, cationic, etc.) is always recommended. A mobile phase containing a high surfactant concentration (10 to 100 times above critical micellar concentration) can be used to quickly saturate the column with the surfactant. Then, 5 to 10 column volumes of this mobile phase are used to rinse the column to equilibrate it.

Micellar phase should never stay motionless in a chromatographic system; this is an essential rule of care. The mobile phase can be recycled. If the operator has to work several days with the same mobile phase, it is possible to keep a micellar phase overnight if the pump is not turned off, decreasing the flow to a minimum value will reduce the pressure and pump wear. It also guarantees the good equilibrium of the adsorbed surfactant in the column.

A cleaning procedure has to be followed to keep the column in good condition. The operator should clean it according to the following steps: the micellar phase has to be replaced by 100% pure water. After rinsing the column with 10 to 20 column volumes of pure water, at least 10 column volumes of 100% methanol is used to remove the adsorbed surfactant in the chromatographic system. Afterwards, the power can be turned off.

On the other hand, when biological fluids are directly injected into the chromatographic system, the surfactant plays an important role. Surfactant micelles tend to bind proteins competitively by releasing protein-bound drugs, meaning that the substances are free to partition into the stationary phase, whereas the proteins, rather than precipitating into the column, are solubilized and eluted with or shortly after the solvent front.

## Results and Discussion

### Optimizing the potential of ED

In order to establish the oxidation potential that gives the best sensitivity for the detection of acetaminophen, the applied potential was varied from 100 to 800 mV. At each voltage, triplicate injections were performed in the selected mobile phase (0.02M SDS at pH 7). Measurements of reten-

tion time, peak area, efficiencies, and asymmetry factors were performed. It was observed that the compounds start to oxidize at potentials above 200 mV, and the maximum peak area values remain unchanged within the 500–800-mV range, though the other chromatographic values were almost the same. Above all, when working with biological samples of serum and urine without any kind of pretreatment, it was advisable to work at the lowest possible potential without losing sensitivity (500 mV) because this reduces the chance of any interfering oxidizable compound having an oxidation potential above 500 mV.

**Influence of pH and SDS concentration**

Acetaminophen is a basic compound (log $K$ = 9.71) with an equilibrium between two forms, one molecular and another positively charged. In the normal pH working range of C18 columns (3–7), the molecular form of acetaminophen is predominant from pH 3–7, and there are no changes in the retention behavior in this pH range. Experimentally, using mobile phases containing 0.05M SDS buffered at pH 7, 5, and 3 showed no changes in the retention time. As pH 7 is more column and environmentally friendly, it was decided that further studies would be carried out using this pH.

Moreover, acetaminophen is a hydrophilic substance (log $P_{o/w}$ = 0.47), meaning that by using C18 columns and micellar mobile phases of SDS, this substance should not be retained very much and ought to appear at short intervals.

The effect of SDS concentration on the separation of acetaminophen from the protein band was studied using mobile phases containing 0.02–0.1M SDS, and the chromatographic parameters *(k*, *N,* and *B/A*) were compared. This study was performed with acetaminophen in micellar solution, and a decrease in the retention times was observed when SDS concentration increased. The retention time only changes by 1 min (4.3 min for 0.02M and 3.3 min for 0.1M) in the concentration range that was studied. There was no marked change in the efficiencies and asymmetries.

The mobile phase that was finally selected was 0.02M SDS, which allows the drug to be analyzed in a short time without any interference with the proteins or endogenous compounds, using only a small amount of surfactant.

**Serum and urine blanks behavior**

When serum and urine samples are injected directly into an MLC system with UV and ED detection, the chromatograms show a band of proteins and endogenous compounds placed in the first few minutes called a protein band, as can be seen in Figure 1. The profile of the protein band is different in the two cases (i.e., serum and urine). In Figures 1A and 1B, the serum protein band is observed to have a similar profile in both detection modes; only a large, well-defined peak appears

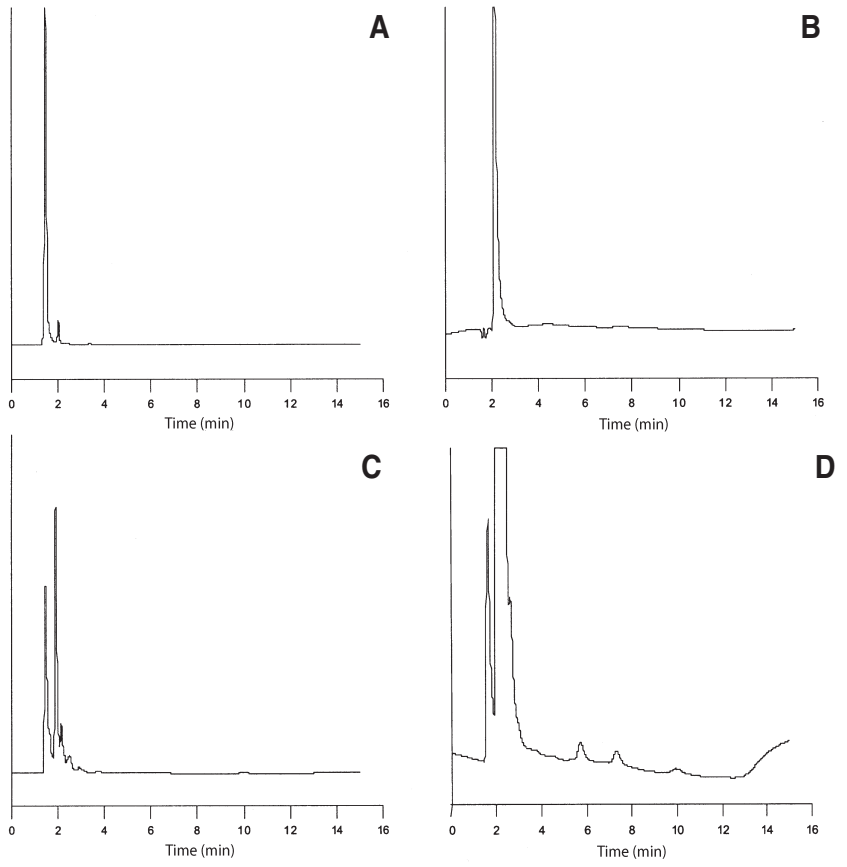


**Figure 1.** Blanks of biological samples of serum (A and B) and urine (C and D) eluted in the selected mobile phase of 0.02M SDS using UV (A and C) and ED (B and D) detection.

**Table I. Calibration Parameters of Acetaminophen**

| Matrix | Detector | Concentration range (µg/mL) | Regression equation* ($n$ = 6) Slope ($b$ ± SD) | Intercept ($a$ ± SD) | $r$ |
|---|---|---|---|---|---|
| Micellar solution | UV | 0.5–50 | 41.5 ± 0.5 | 0.18 ± 0.06 | 0.9998 |
| | ED | 0.05–5 | 220 ± 2 | 1.012 ± 0.013 | 0.9998 |
| Serum | UV | 0.5–50 | 40.0 ± 0.3 | –1.12 ± 0.16 | 0.9989 |
| | ED | 0.05–5 | 215.0 ± 1.2 | 0.5 ± 0.3 | 0.9996 |
| Urine | UV | 0.5–50 | 36.02 ± 0.13 | 0.20 ± 0.18 | 0.9992 |
| | ED | 0.05–5 | 202.0 ± 0.3 | 1.10 ± 0.08 | 0.9994 |

* Injections were made by triplicate.

at 1.5 min in UV and at 2.5 min in ED. On the other hand, Figures 1C and 1D show different profiles. When UV is used, several peaks appear at the beginning of the chromatogram, and in ED the protein band is wider because there are many compounds that can be oxidized at this voltage.

### Calibration and linearity

Calibration curves were constructed using the areas of the chromatographic peaks measured at six increasing concentrations in three different matrices: micellar solution, urine, and serum. Table I shows the ranges of concentration, slopes, intercepts, and regression coefficients of the calibration curves obtained using UV and ED detection.

**Table II. Intra- and Interday Precision (RSD, %) at Three Different Concentrations**

| | | Intraday ($n$ = 10) | | | Interday ($n$ = 5) | | |
|---|---|---|---|---|---|---|---|
| Matrix | Detector | $c_1$ | $c_2$ | $c_3$ | $c_1$ | $c_2$ | $c_3$ |
| Serum | UV* | 4.1 | 2.8 | 1.1 | 3.0 | 3.9 | 1.8 |
| | ED† | 3.6 | 2.5 | 1.2 | 4.1 | 3.5 | 0.86 |
| Urine | UV* | 4.3 | 3.1 | 1.7 | 4.9 | 2.8 | 2.1 |
| | ED† | 3.1 | 5.2 | 1.0 | 2.2 | 1.0 | 0.75 |

* For UV: $c_1$ = 1, $c_2$ = 20, and $c_3$ = 45 µg/mL.
† For ED: $c_1$ = 0.1, $c_2$ = 1, and $c_3$ = 4 µg/mL for ED.

### Intra- and Interday precision

The intraday precision of the method was determined by preparing spiked serum and urine samples at three different concentrations of acetaminophen within the calibration range. Ten replicates were performed at each concentration. Interday precision was checked with the same concentrations as in the intraday assay, and the determination was repeated daily for 5 days. The results are shown in Table II and the relative standard deviation (RSD) was always below 5.2%.

**Table III. LOD and LOQ for Acetaminophen in Different Matrices**

| Matrix | Detector | LOD (ng/mL) | LOQ (ng/mL) |
|---|---|---|---|
| Micellar solution | UV | 10.1 | 35 |
| | ED | 0.56 | 2.8 |
| Serum | UV | 16.1 | 40 |
| | ED | 0.83 | 4.4 |
| Urine | UV | 13.8 | 45 |
| | ED | 0.74 | 5.8 |

### Limits of detection and quantitation

Limits of detection (LOD) (3s criterion) were evaluated by injecting a series of 10 solutions containing acetaminophen at the lowest concentration of the calibration curve. Limits of quantitation (LOQ) were calculated following the 10s criterion. Results are shown in Table III.

The values of the LODs using UV detection were similar to those typically reported in the literature, and they were 20 times higher than when ED is used. These values enable detection and quantitation of acetaminophen in biological matrices using the proposed method, with the advantage that the biological samples were injected without any previous treatment. The ED detector allows the pharmacokinetics of the drug to be studied.

**Table IV. Determination of Acetaminophen* in Spiked Serum and Urine Samples Using 0.02M SDS at pH 7**

| Matrix | Detector | Added | Found |
|---|---|---|---|
| Serum | UV | 20 | 21.2 ± 1.1 |
| | | 10 | 9.8 ± 0.7 |
| | | 5 | 5.1 ± 0.8 |
| | ED | 3 | 3.03 ± 0.12 |
| | | 2 | 1.95 ± 0.18 |
| | | 1 | 1.05 ± 0.09 |
| Urine | UV | 20 | 21.0 ± 1.4 |
| | | 10 | 10.8 ± 1.2 |
| | | 5 | 5.02 ± 0.19 |
| | ED | 3 | 3.2 ± 0.2 |
| | | 2 | 2.05 ± 0.08 |
| | | 1 | 0.98 ± 0.13 |

* In µg/mL, mean ± SD, $n$ = 5.

### Analysis of serum and urine samples

The applicability of the method developed to determine acetaminophen was verified by its analysis in both spiked and real serum and urine samples. The results shown in Table IV indicate that determination in biological samples is possible at therapeutic levels following the administration of pharmaceuticals containing acetaminophen. An ED detector is useful to determine the drug at low concentrations, taking into account that the biological samples are injected after being diluted 10 times with micellar solution. Recoveries obtained were from 98% to 107%.

Figure 2 shows the chromatograms for acetaminophen in spiked serum and urine samples using an ED detector at LOQ concentration. No interference was observed, and the acetaminophen peak was separated from the protein band large enough to be quantitated.

The analysis time in the developed method was less than 5 min, which makes it faster and easier to use than those using aqueous–organic mobile phases that need previous extraction steps.

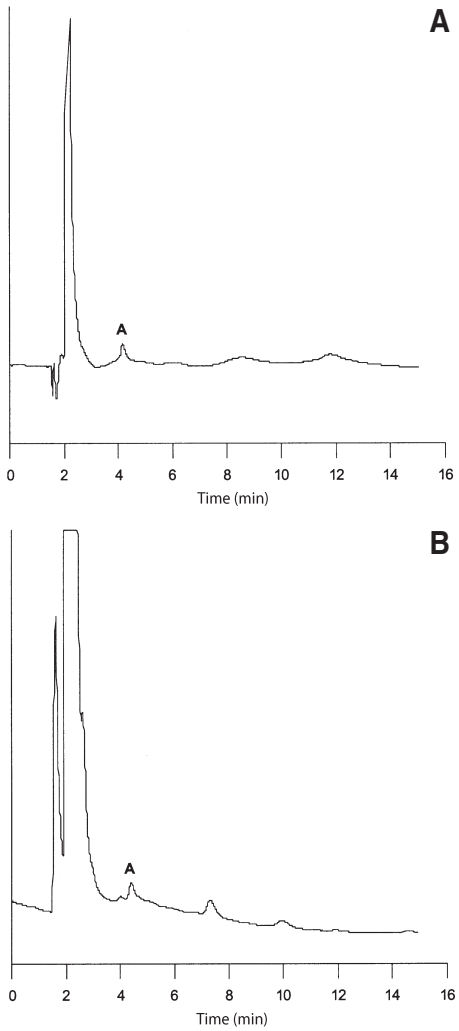


**Figure 2.** Determination of acetaminophen in biological samples, serum (A) and urine (B), using ED detection at 500 mV and LOQ concentrations of the drug. (A = acetaminophen.)

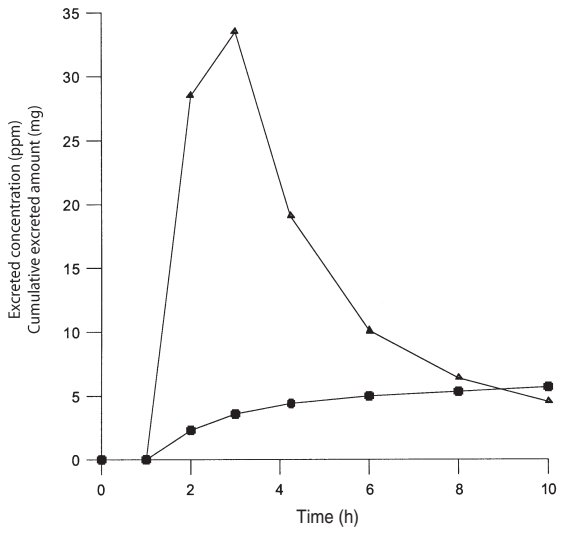


**Figure 3.** Elimination curve (▲) and cumulative excreted curve (•) of acetaminophen in urine.

**Drug elimination in urine and distribution in serum**

Following oral administration of a single conventional 500-mg tablet to 5 normal healthy volunteers, urinary and serum studies were conducted. A sample was collected just before administration for use as the blank. Samples of urine were taken over 10 h at different time intervals, and the volume was measured. Serum samples followed for 24 h. Dilution of 1:10 of biological samples in 0.02M SDS was performed.

Figure 3 depicts the elimination curve of acetaminophen in urine; the maximum concentration excreted as unchanged drug occurs at 3 h after ingestion. The cumulative excretion curve shows the final quantity of acetaminophen eliminated (i.e., 10% of the dose taken). Figure 4 shows the chromatogram of acetaminophen excreted in urine 3 h after oral ingestion. Two peaks are observed: acetaminophen as unchanged drug and a second peak of a metabolite at 10 min. This peak does not appear in the urine blank sample. It appears 2 h after ingestion and continues throughout the interval studied, with the maximum concentration of the metabolite being observed 5 h after oral administration. It is known that at therapeutic doses, acetaminophen is metabolized to non-toxic glucuronide and sulfate conjugates.

Serum concentration of acetaminophen was monitored for 24 h at different intervals in five healthy volunteers. The half-life obtained, using the developed method, was 2.2 h. It was also found that the maximum concentration of acetaminophen in serum occurs 2 h after oral ingestion.

## Conclusion

The main advantage of the method developed here is the possibility of direct injection of biological (serum and urine) sam-

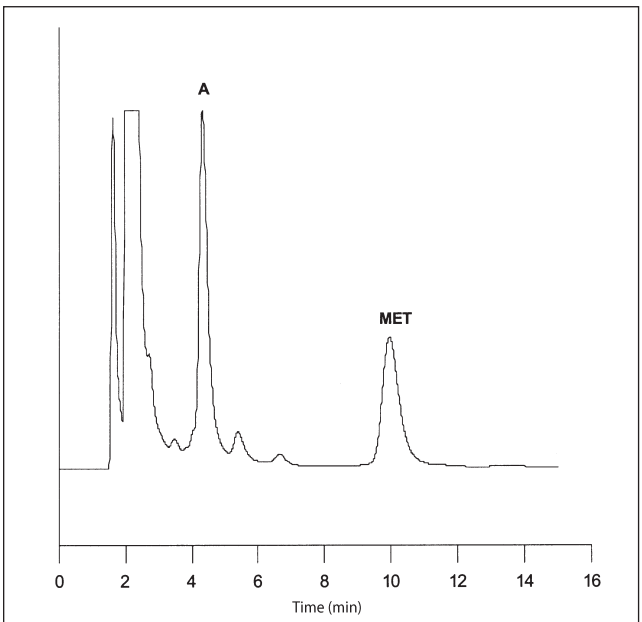


**Figure 4.** Chromatogram of acetaminophen excreted in urine 3 h after oral ingestion; a second peak of a metabolite can be observed at 10 min (A = acetaminophen and MET = metabolite).

ples by solubilizing the proteins and other components of the matrix. Another advantage is the short analysis time; acetaminophen is eluted in less than 5 min in a mobile phase of 0.02M SDS without any organic modifier. The ED detector is well-suited to follow the excretion and distribution of acetaminophen in biological samples.

Spiked acetaminophen in serum and urine agreed with the injected quantity, and recoveries were close to the concentration added. Finally, results of the method were compared with those obtained using a conventional aqueous–organic HPLC method with a previous extraction step, and similar results were obtained. The procedure reported here could be used in routine analyses, taking into account that it is quick, sensitive, precise, and useful for the determination of acetaminophen in biological fluids (serum and urine) without any previous treatment. The pure SDS micellar mobile phases used here have the advantage of having lower toxicity, flammability, and environmental impact in comparison with conventional HPLC that uses organic solvents.

## Acknowledgments

This work was funded by Project BQU2001-3770 (MCYT) and P1-1B2003-07 (Bancaixa), and Conselleria d'Educació i Ciència de la Generalitat Valenciana provided fellowships for Dr. D. Bose and Dr. A. Durgbanshi, respectively.

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Manuscript received September 29, 2004;
revision received May 5, 2005.