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# Spectrofluorimetric determination of paracetamol in pharmaceuticals and biological fluids

José Luis Vilchez, Rosario Blanc, Ramiro Avidad, Alberto Navalón *

*Departamento de Química Analítica, Universidad de Granada, E-18071 Granada, Spain*

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**Abstract**

A spectrofluorimetric method for the determination of paracetamol is presented, based on the oxidation of the analyte to give the fluorophore 2,2'-dihydroxy-5,5'-diacetyldiaminebiphenyl. Sodium hypochlorite was used as an oxidizing reagent and the optimum pH was found to be 10.0 (sodium carbonate–boric acid buffer solution). The linear concentration range of application was 0.1–100.0 μg ml$^{-1}$ of paracetamol, the detection limit 0.01 μg ml$^{-1}$ and the relative standard deviation 1.2%. The method has been satisfactorily applied to the determination of paracetamol in pharmaceutical formulations and biological fluids.

*Keywords:* Spectrofluorimetry; Paracetamol; Pharmaceuticals; Biological fluids

## 1. Introduction

Paracetamol (*N*-acetyl-4-aminophenol) is widely used as an active ingredient in pharmaceutical preparations. This substance is mainly used as an alternative to aspirin because of its analgesic and antipyretic activity. Analytical tests for paracetamol have been discussed in reviews [1,2].

Several analytical procedures using a separatory technique such as TLC [3–5], HPLC [6–9] and GC [10,11] or the use of an ion-exchange resin [12] have been proposed for the determination of paracetamol. Spectrophotometric determinations for paracetamol in combination with caffeine [13] have also been described, as well as for paracetamol with salicylamide or with oxyphenbutazone and salicylamide through nitrosation and subsequent chelation [14,15]. However, the majority of these methods require lengthy treatments and are not suitable for routine analysis. Voltamperometric techniques have also been proposed for the determination of paracetamol in analgesic products [16].

Spectrofluorimetric methods with lower detection limits have been proposed for the determination of paracetamol in binary or ternary mixtures of drugs in pharmaceutical formulations [17–21]. Because paracetamol is not a fluorescent species, it can be determined indirectly using Ce(IV) [17] as an oxidizing reagent and measuring the relative fluorescence intensity of Ce(III) arising from Ce(IV). Direct spectrofluorimetric determinations of paracetamol require a previous and adequate derivatization step. Reagents such as fluorescamine and dansyl chloride have been proposed [18,19] but both reactions show low selectivity. 1-Nitroso-2-naphthol [20] and potassium hexacyanoferrate(III) [21] have been proposed as oxidizing reagents, since an adequate oxidation of paracetamol produces fluorogenic species suitable for its determination.

In this paper, an easier and quite sensitive spectrofluorimetric method is proposed for the determination of paracetamol, whereby sodium

* Corresponding author.


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hypochlorite is used as an oxidizing reagent yielding the fluorophore 2,2′-dihydroxy-5,5′-diacetyldiaminebiphenyl. The procedure has been applied satisfactorily to the determination of paracetamol in different pharmaceutical products and biological fluids.

## 2. Experimental

### 2.1. Reagents

All the experiments were performed with analytical-reagent grade chemicals and pure solvents. Double-distilled demineralized water was used.

*Paracetamol stock solution, 1.00 mg ml$^{-1}$*

This was prepared by dissolution of the reagent (Aldrich-Chemie, Steinheim, Germany) in double-distilled water. The solution was stable for at least 1 week. Working solutions were obtained by appropriate dilution with double-distilled water.

*Sodium hypochlorite solutions, $10^{-2}$, $10^{-3}$, $5 \times 10^{-3}$ and $5 \times 10^{-4}$ M*

These were prepared by dilution of a 70.0 g l$^{-1}$ solution (Sigma, St Louis, MO) in double-distilled water.

*Buffer solutions*

Solutions of the required pH were prepared from 0.4 M $Na_2CO_3$ (Merck, Darmstadt, Germany) and 0.4 M $H_3BO_3$ (Merck), and from 0.1 M sodium acetate and 0.1 M acetic acid.

*β-Glucuronidase/arylsulphatase solution*

A commercial preparation (Boehringer Mannheim Gmbh, Germany) from *Helix pomatia*, approximately 5.2 U ml$^{-1}$ (with phenolphthalein monoglucuronide as a substrate) of β-glucuronidase and 2.6 U ml$^{-1}$ (with phenolphthalein disulphate as a substrate) of arylsulphatase, was diluted 40 times with 0.1 M acetic acid–acetate buffer (pH 5.0).

### 2.2. Samples of biological fluids

Plasma and urine samples were obtained from healthy volunteers who had received a single oral dose of paracetamol (650 mg). Whole blood samples were collected 30–45 min after administration. Urine samples were collected for 24 h after administration of paracetamol and the urinary volumes were recorded.

### 2.3. Apparatus

All spectrofluorimetric measurements were performed using a Perkin-Elmer LS-5 luminescence spectrometer, equipped with a xenon discharge lamp (9.9 W) pulsed at line frequency, Monk-Gillieson F/3 monochromators, a Rhodamine 101 counter to correct the excitation spectra, a Hamamatsu R928 photomultiplier, a Houston Omnigraphic $x$–$y$ recorder and a Braum Melsungen Thermomix 1441 thermostat. In order to compare all the spectrofluorimetric measurements and ensure reproducible experimental conditions, the LS-5 spectrometer was checked daily with a fluorescent polymer standard of $p$-terphenyl ($10^{-7}$ M) having a relative fluorescence intensity of 90% when measured at a wavelength of maximum emission ($\lambda_{em}$) of 340 nm and a wavelength of excitation ($\lambda_{ex}$) of 295 nm, excitation and emission slit-widths of 2.5 nm and a sensitivity factor of 0.594.

The LS-5 spectrometer was interfaced with an IBM PS/2 30-286 microcomputer, with RS 232C connections for spectral acquisition and subsequent manipulation of spectra as described previously [22]. A Canon BJ-300 printer was used for graphical representation.

A Crison 501 digital pH-meter with a combined glass-saturated calomel electrode was also used.

### 2.4. Fluorescence measurements

The relative fluorescence intensity (RFI) of the solution containing the fluorescent product was measured in a standard $1.0 \times 1.0$ cm quartz cell at $20.0 \pm 0.5$ °C.

### 2.5. Basic procedure

To a 50-ml calibrated flask containing 5–500 μg of paracetamol, 10 ml of 0.4 M $Na_2CO_3$–$H_3BO_3$ buffer solution (pH 10.0) and 3.5 ml of $10^{-3}$ M sodium hypochlorite were added, and the mixture was diluted with double-distilled water to approximately 45 ml and heated at 80 °C for 2 min. After cooling to 20 °C, the volume was adjusted with double-distilled water to 50 ml. A reagent blank solution was prepared in a similar way. The fluorescence intensities of the sample and the blank were always measured at $\lambda_{em} = 427$ nm

with $\lambda_{ex} = 335$ nm. A calibration graph was constructed in the same way using paracetamol solutions of known concentration.

If the amount of paracetamol is 0.5–5 mg, the concentration of sodium hypochlorite solution must be $10^{-2}$ M.

### 2.6. Procedure for the determination of paracetamol in formulations

A sample containing 100–700 mg of paracetamol was introduced into a 1-l calibrated flask and diluted to the mark with double-distilled water. After filtering through Whatman No. 1 filter paper, 5 ml of the filtrate was mixed with 10 ml of 0.4 M $Na_2CO_3$–$H_3BO_3$ buffer solution (pH 10.0) and 3.5 ml of $10^{-2}$ M sodium hypochlorite. The mixture was diluted with double-distilled water to about 45 ml and heated at 80 °C for 2 min. After cooling, the solution was diluted to 50 ml with double-distilled water. The determination was carried out as in Section 2.5.

In formulations containing salicylic acid and/or acetylsalicylic acid, prior extraction of these compounds with ethyl ether in acid medium is necessary. For this purpose, the sample solution (5 ml) is treated with 1 ml of 1 M HCl, transferred into a 100-ml separating funnel and shaken with 10 ml of ethyl ether for 10 min. Paracetamol is determined in the aqueous phase as indicated above.

### 2.7. Procedure for the determination of free paracetamol in human urine and plasma

Double-distilled water was added to biological samples (0.5 ml of plasma or 1 ml of urine) to adjust the volume to 3 ml. After the addition of 2 M NaOH to pH ≈ 10 (1–2 drops), the mixture was transferred into a 100-ml separating funnel and shaken with 10 ml of ethyl acetate for 10 min. The organic layer was filtered through Whatman No. 1 filter paper, transferred to a glass-stoppered tube and the ethyl acetate was evaporated to dryness under $N_2$ using a sample evaporator. The residue was redissolved in 5 ml of 0.4 M $Na_2CO_3$–$H_3BO_3$ buffer solution (pH 10.0) and transferred to a 25-ml calibrated flask, to which 3.5 ml of $5 \times 10^{-4}$ M sodium hypochlorite was added. The mixture was diluted with double-distilled water to about 20 ml and heated at 80 °C for 2 min. After cooling, the solution was diluted with double-distilled water to 25 ml. The subsequent steps were as in Section 2.5.

### 2.8. Procedure for the determination of the sum of free, glucuronidated and sulphated paracetamol in human urine and plasma

Samples of 0.5 ml of plasma or 1 ml of urine were treated with 0.5 ml of β-glucuronidase arylsulphatase solution and an appropriate volume of 0.1 M acetic acid–acetate buffer solution (pH 5.0) to adjust the volume to 3 ml. The mixture was incubated at 37 °C for 24 h and then 1–2 drops of 2 M NaOH were added to adjust the pH to about 10 [21]. The mixture was transferred to a 100-ml separating funnel and shaken with 10 ml of ethyl acetate for 10 min. The organic layer was filtered through Whatman No. 1 filter paper, transferred to a glass-stoppered tube and the ethyl acetate was evaporated to dryness under $N_2$ using a sample evaporator. The residue was redissolved in 5 ml of 0.4 M $Na_2CO_3$–$H_3BO_3$ buffer solution (pH 10.0) and transferred to a 25-ml calibrated flask. Then, 3.5 ml of $5 \times 10^{-3}$ M or 3.5 ml of $5 \times 10^{-4}$ M sodium hypochlorite were added for urine or plasma samples, respectively. The subsequent steps were as in Section 2.7.

## 3. Results and discussion

### 3.1. Spectral characteristics

Paracetamol can be oxidized by an oxidizing agent such as sodium hypochlorite at pH ≈ 10 to form 2,2′-dihydroxy-5,5′-diacetyldiaminebiphenyl [21] (Fig. 1). This compound shows native fluorescence with an excitation maximum at $\lambda_{exc} = 335$ nm and an emission maximum at $\lambda_{em} = 427$ nm (Fig. 2).

### 3.2. Effect of experimental variables

In order to find the best agent for the oxidation of paracetamol to the fluorophore 2,2′-di-

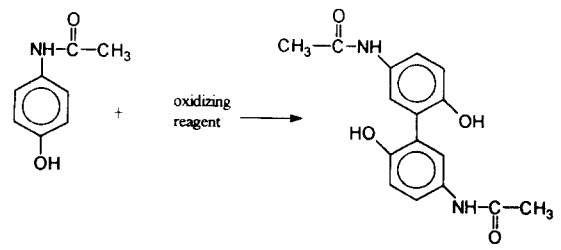


Fig. 1. Oxidation of paracetamol.

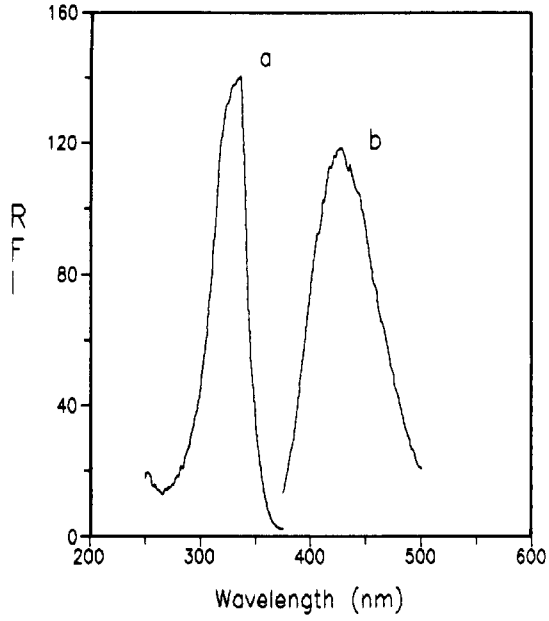


Fig. 2. Fluorescence spectra of 2,2′-dihydroxy-5,5′-diacetyldiaminebiphenyl: (a) excitation, (b) emission.

hydroxy-5,5′-diacetyldiaminebiphenyl, the effects of potassium hexacyanoferrate(III), hydrogen peroxide and sodium hypochlorite were compared. It was found that hypochlorite is to be preferred because the relative fluorescence intensity is higher than for the other oxidizing agents. Fig. 3 shows the behaviour of paracetamol in the presence of these oxidizing agents at different pH values. The highest value of RFI at pH 10.0 using sodium hypochlorite is clearly observed.

Different buffer solutions (TRIS–HCl, $Na_2HPO_4$–$NaH_2PO_4$ and $Na_2CO_3$–$H_3BO_3$) were tested; 0.4 M $Na_2CO_3$–$H_3BO_3$ (pH 10.0) was found to be the best. A concentration of 0.08 M of this buffer solution was then selected to obtain an adequate buffering capacity. In every experiment, the RFI increased slowly, requiring some 30 min to stabilize before carrying out the measurements. This waiting time could be eliminated (and the RFI was considerably increased) if the solution was heated at 80 °C for 2 min. The RFI was measured after cooling and remained constant for at least 24 h.

The fluorescence was shown to be independent of ionic strength, adjusted in buffered solutions with up to 2 M NaCl or $NaClO_4$.

Tests on the influence of hypochlorite concentration in the oxidation of $2.65 \times 10^{-4}$ M paracetamol showed that the RFI is independent of hypochlorite concentration in the range $5 \times 10^{-4}$–$10^{-3}$ M. The RFI decreased slightly at higher concentrations of oxidizing reagent. A concentration of $7 \times 10^{-4}$ M of hypochlorite was selected as adequate.

Since in the temperature range 5–70 °C the RFI was found to be practically independent of the temperature, all measurements reported here were made at 20.0 ± 0.5 °C.

The order of addition of reagents did not affect the results obtained. The order used in the proposed method was paracetamol, buffer and hypochlorite.

### 3.3. Analytical parameters

Under the recommended conditions, there is a linear relationship between the analytical signal (RFI) and paracetamol concentration ($C$) over the range 0.1–10.0 μg ml$^{-1}$ (RFI = 0.1 + 41.2$C$ ($r = 0.9995$, $n = 9$)) and the range 10.0–100.0 μg ml$^{-1}$ (RFI = 0.2 + 2.4$C$ ($r = 0.9997$, $n = 10$)). The repeatability of the proposed method was checked with two series of 10 samples having a paracetamol concentration of 5.0 μg ml$^{-1}$ and 40.0 μg ml$^{-1}$, respectively. The relative standard deviation (RSD) ($P = 0.05$, $n = 10$) was 1.2% in both cases. The precision (RSD) of the fluorescence measurements (noise) was about 0.5% in all instances. The IUPAC detection limit ($k = 3$) [23] was 0.01 μg ml$^{-1}$ and the quantification limit ($k = 10$) [24] was 0.03 μg ml$^{-1}$.

The proposed method was compared with methods described in the literature for the spectrofluorimetric determination of paracetamol. For comparison purposes, those methods that were considered to be among the most sensitive reported to date were selected (Table 1).

### 3.4. Interference

The effect of species and ions commonly found in paracetamol formulations and/or biological fluids as potential interferents was studied on the determination of paracetamol at the 40.0 μg ml$^{-1}$ level. A 150 μg ml$^{-1}$ level of each potentially interfering species was tested first, and, if interference occurred, the ratio was reduced progressively until interference ceased. Tolerance was defined as the amount of foreign species that produced an error not exceeding ±5% in the determination of the analyte. The results obtained are summarized in Table 2.

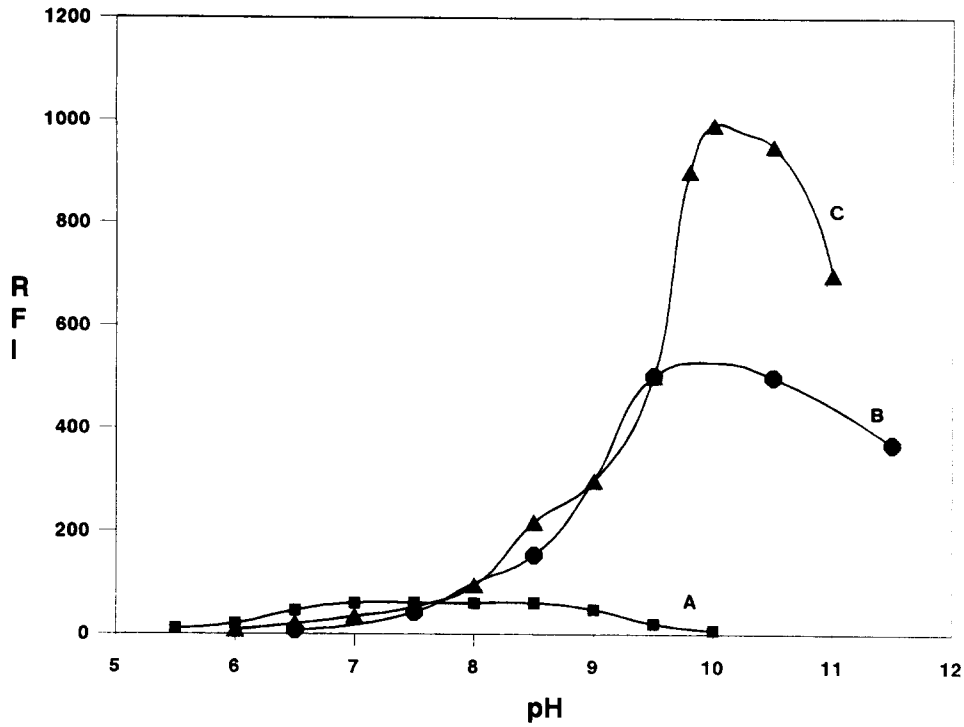


Fig. 3. Influence of oxidizing reagent and pH on paracetamol. [Paracetamol] = $8 \times 10^{-4}$ M. (A) [$H_2O_2$] = $4 \times 10^{-4}$ M. (B) [$K_3[Fe(CN)_6]$] = $8 \times 10^{-4}$ M. (C) [NaOCl] = $8 \times 19^{-4}$ M.

The most serious interference was from salicyclic acid and acetylsalicylic acid. These sources of interference could be removed by previous extraction of the interfering species with ethyl ether as indicated in the procedure.

It is well known that the major biotransformation of paracetamol in man and in most animals is conjugation with glucuronic acid and sulphuric acid, while deacetylation is relatively minor [21,25].

In order to test the potential interference from glucuronidated paracetamol and sulphated paracetamol in the determination of free paracetamol in biological fluids, the proposed method was applied to plasma and urine samples with and without enzyme treatment ($\beta$-glucuronidase–arylsulphatase solution for hydrolysis of these conjugates) [21]. The results obtained were compared with those obtained using the Syva EMIT acetaminophen immunoassay [26]; good agreement was obtained in all instances (see Table 5).

The inhibitory effect of *p*-aminophenol (another metabolite of paracetamol) was investigated and found to be rather large at higher levels, but negligibly small at levels up to three times that of paracetamol involved in the present work.

Thus, the present method allows the determination of free paracetamol in human biological fluids without interference by *p*-aminophenol

Table 1
Methods for the spectrofluorimetric determination of paracetamol

| Reagent | $\lambda_{exc}/\lambda_{em}$ (nm) | Detection limit ($\mu g\ ml^{-1}$) | Reference |
|---|---|---|---|
| $K_3[Fe(CN)_6]$ | 337/427 | 4 | [21] |
| 1-Nitroso-2-naphthol | 467/552 | 2 | [20] |
| Fluorescamine | 390/480 | 1.5 | [18] |
| NaOCl | 335/427 | 0.01 | This work |
| Dansyl chloride | 365/530 | 0.005 | [19] |

Table 2
Interference from other ions or species

| Foreign ion or species | Tolerance level ($\mu g\ ml^{-1}$) |
|---|---|
| $Na_3PO_4$, aniline, *p*-aminophenol, phenacetin, acetanilide | >150 |
| Citric acid | 140 |
| Caffeine, Ca(II) | 50 |
| Ascorbic acid, Mg(II) | 35 |
| Codeine, Al(III), Fe(III) | 25 |
| Salicylic acid | 8 |
| Acetylsalicylic acid | 5 |

Table 3
Recovery study of paracetamol in biological fluids

| Biological fluid | Paracetamol | | |
|---|---|---|---|
| | Added ($\mu g\ ml^{-1}$) | Found [a] ($\mu g\ ml^{-1}$) | |
| | | Proposed method | EMIT [b] |
| Urine | 10.0 | 9.9 ± 0.2 | 9.8 ± 0.9 |
| | 15.0 | 15.2 ± 0.3 | 15 ± 1 |
| | 20.0 | 19.7 ± 0.3 | 19 ± 2 |
| Plasma | 10.0 | 9.8 ± 0.2 | 10 ± 1 |
| | 15.0 | 14.8 ± 0.3 | 15 ± 1 |
| | 20.0 | 19.8 ± 0.3 | 21 ± 2 |

[a] Mean values ± standard deviation of six determinations.
[b] Enzyme-multiplied immunoassay (Syva Co. Inc., Palo Alto, CA).

and its conjugates (paracetamol glucuronide and paracetamol sulphate).

### 3.5. Recovery study

In order to check the accuracy of the proposed method, a recovery study was carried out on both drug-free urine and plasma samples. For this, various amounts of paracetamol were added to samples and the percentage recovery was determined. Table 3 shows the results obtained.

### 3.6. Applications

The proposed method was applied to commercial paracetamol formulations of the

Table 4
Determination of paracetamol in drug formulations

| Proprietary name | | Composition [a] (%) | Found [b] (%) | Recovery (%) |
|---|---|---|---|---|
| Gelocatil (*Gelos S.A.*) | Paracetamol | 65.0 | 66 ± 1 | 101.5 |
| Efferalgan (*Upsamédica S.A.*) | Paracetamol | 17.0 | 17.3 ± 0.3 | 101.7 |
| | Saccharin | 0.2 | | |
| Efferlagan (*Upsamédica S.A.*) | Paracetamol | 11.0 | 11.2 ± 0.3 | 101.8 |
| | Ascorbic acid | 7.0 | | |
| Termalgin (*Sandoz, S.A.E.*) | Paracetamol | 71.5 | 71 ± 1 | 99.3 |
| Tylenol (*Johnson & Johnson*) | Paracetamol | 83.0 | 83 ± 1 | 100.0 |
| Hemicraneal (*Liade S.A.*) | Paracetamol | 50.0 | 49.2 ± 0.8 | 98.4 |
| | Caffeine | 17.0 | | |
| | Ergotamine | 0.2 | | |
| Algidol [d] (*Berenguer Beneyto S.A.*) | Paracetamol | 12.0 | 12.2 ± 0.3 | 101.7 |
| | Ascorbic acid | 9.0 | | |
| | Codeine | 0.2 | | |
| Actron [c] (*Miles-Martin*) | Parcetamol | 4.5 | 4.4 ± 0.1 | 97.8 |
| | Aspirin | 9.0 | | |
| | Caffeine | 1.0 | | |
| | Sodium bicarbonate | 53.0 | | |
| | Citric acid | 32.0 | | |
| Frenadol [d] (*Abelló S.A.*) | Paracetamol | 6.0 | 6.1 ± 0.1 | 101.7 |
| | Salicylamide | 1.0 | | |
| | Codeine | 0.1 | | |
| | Caffeine | 0.3 | | |
| | Chlorphenamine | 0.04 | | |
| | Ascorbic acid | 5.0 | | |

[a] Indicated by the suppliers.
[b] Mean values ± standard deviation of six determinations.
[c] In this case, a prior extraction with ethyl ether was carried out. The paracetamol remained in aqueous solution.
[d] In these instances, the amount of oxidizing agent is double that in the other cases.

Table 5
Determination of paracetamol in biological fluids

| Biological fluid | Paracetamol found [a] (μg ml$^{-1}$) | | | |
|---|---|---|---|---|
| | Without enzyme treatment | | With enzyme treatment | |
| | Proposed method | EMIT [b] | Proposed method | EMIT [b] |
| Plasma 1 | 135 | 130 | 133 | 134 |
| Plasma 2 | 122 | 120 | 123 | 121 |
| Plasma 3 | 130 | 128 | 128 | 132 |
| Plasma 4 | 125 | 124 | 127 | 125 |
| Urine 1 | 21 | 20 | 1050 | 1048 |
| Urine 2 | 15 | 14 | 435 | 434 |
| Urine 3 | 16 | 15 | 670 | 668 |
| Urine 4 | 19 | 20 | 960 | 965 |

[a] Data are the average values of three determinations.
[b] Enzyme-multiplied immunoassay (Syva Co. Inc., Palo Alto, CA).

Spanish Pharmacopoeia. Samples were treated and analyzed as described in the Experimental section. The results obtained, summarized in Table 4, show good agreement with the composition indicated by the suppliers. A voltammetric method based on the oxidation of paracetamol at a glassy carbon electrode using an aqueous acetic acid–acetate buffer [16] was used as a comparison method. Both methods (spectrofluorimetric and voltammetric) yield values within the same range when tested using adequate statistical procedures [27].

The method was also applied to the determination of free paracetamol and the sum of free paracetamol, paracetamol glucuronide and paracetamol sulphate in human urine and plasma. Samples were treated and analyzed as described in the experimental section. In this case, the Syva EMIT acetaminophen immunoassay [26] was used as a reference method. The results obtained are summarized in Table 5.

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