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A Highly Selective and Sensitive Spectrofluorimetric Method for the Determination of *N*-acetyl-4-aminophenol at Nano-trace Levels in Pharmaceuticals and Biological Fluids Using Cerium(IV)

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Abstract

A very simple, rapid, ultra-sensitive, highly selective and non-extractive spectrofluorimetric method for the determination of *N*-acetyl-4-aminophenol (paracetamol) at ultra-trace levels has been developed. This method was based on the oxidation of paracetamol in presence of slightly acidic (0.05 – 0.15 M H_2SO_4) aqueous solution with a prompt oxidizing agent, cerium(IV) for the direct spectrofluorimetric determination of paracetamol and the fluorescent species is an oxidation product of parcetamol, has excitation and emission wavelength at λ_{ex} = 255 nm and λ_{em} = 350 nm, respectively. The fluorescence intensity of oxidation product reaches a constant value (after heating for 5 min at 45 ± 5°C) within 15 min remains stable for over 24 h. Numerous variables influencing the reaction's conditions e.g. the concentrations of cerium(IV), temperature, effect of acidity, time of the reaction and solvents were cautiously experimented and optimized. Linear calibration graphs were obtained for 10–700 μgL^{-1} of paracetamol, having a detection limit of 2 μgL^{-1}; the quantification limit of the reaction system was found to be 10 μgL^{-1}; the RSD was 0 – 2 % and the correlation coefficient, R^2 = 0.9999. A large excess of over 40 potentially interfering excipients, commonly present in dosage forms were tested in the determination of paracetamol at 100 μgL^{-1} level, do not intervene in the determination process. The developed method was successfully applied to the determination of paracetamol in commercial pharmaceutical formulations and biological fluids. The results of the proposed method for pharmaceuticals and biological analyses were analogous with that of spectrophotometric method and the Official method stated in the British Pharmacopoeia, and was found to be in an excellent agreement.

Keywords: Spectrofluorimetry, Paracetamol determination, Pharmaceutical formulations, Biological fluids

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Introduction

Paracetamol (acetaminophen or *N*-acetyl-4-aminophenol) is a pain-mitigator and a fever-allayer. Paracetamol (PR) is a popular antipyretic and analgesic agent. In several countries, it is one of the most used medicines as an alternative to aspirin (acetylsalicylic acid). It is ordinarily used as an anodyne in pharmaceutical formulations thoroughly or in the mixtures with a little amount of contaminants, typically caffeine, acetylsalicylic acid and few others. PR is used to treat many conditions such as headache, muscle aches, arthritis, backache, toothaches, colds, and fevers. It relieves pain in mild arthritis but has no effect on the underlying inflammation and swelling of the joint [1]. However, overdosing of paracetamol causes liver damage which may lead to patients'

death upon delayed treatment. To provide accurate diagnosis and fast treatment of PR poisoning, rapid analysis of PR in patient tissues is necessary. Following oral administration and absorption from the gastrointestinal tract, PR enters the blood, is distributed throughout the body and metabolized in the liver [2]. It cannot be used more of this medication than is recommended. An overdose of PR can cause serious harm. The maximum amount for adults is 1 gram (1000 mg) per dose and 4-grams (4000 mg) per day [3]. Usual pediatric dose for fever (oral / rectal) ≤ 1 month: 10 to 15 $mgkg^{-1}$ per dose every 6 to 8 hours as needed, and > 1 month to 12 years: 10 to 15 $mgkg^{-1}$ per dose every 4 to 6 hours as needed (Maximum: 5 doses in 24 hours) [3]. At therapeutic doses, PR is largely converted to inactive metabolites by conjugation with sulfate or glucuronide and excreted within 24 hours [3]. PR toxicity is likely to occur after a minimum ingestion of $140mgkg^{-1}$ [3]. At carcinogenic doses liver damage may take place within 24 to 48 hours requiring fast reporting of patients' exposure levels, thus, rapid analysis of PR in biological matrices is of great importance for clinical and forensic toxicologists [3]. Thus, determination of PR in pharmaceuticals (quality control) and in biological fluids (overdose monitoring) is of PR interest.

Numerous analytical methods were reported for the determination of PR in pharmaceuticals such as FIA [4], TLC [5 - 7], GC [8,9], HPLC [10 - 13], Spectrophotometry [14 - 21], Spectrofluorimetry [22 - 39], Voltamperometry [40], Titrimetry [41, 42], UV-Vis spectrophotometry [43 - 46], Near infrared transmittance spectroscopy [47] and Electrochemical methods [48,49] have been applied to the determination of PR which were recently reviewed [50]. Most of the recent methods for PR determination have good accuracy and specificity but they are time consuming, technically demanding, and also require the use of costly, highly specialized instruments and interfering of foreign ions during all the steps of an analytical procedures. In expanding analytical fields such as environmental, biological and medicinal monitoring of drugs, there is an increasing need to develop the simple, sensitive and selective analytical techniques that do not use expensive or complicated test equipments.

Spectrofluorimetric methods with lower detection limits have been proposed for the determination of PR in binary or ternary mixture of drugs in pharmaceutical formulations [22-36]. The aim of this study was to develop a simpler direct spectrofluorimetric method for the ultra-trace determination of PR. The oxidation reaction of PR with Ce(IV) forms an intensely fluorescent oxidized product. This method is basically founded on the oxidative reaction of non-fluorescent PR in a wee acidic (0.05 – 0.15 M H_2SO_4) solution with Ce(IV) in presence of water to generate an extremely fluorescent oxidized product, followed by a forthright measurement of the fluorescence intensity in the aqueous solution at room temperature. The method possesses significant advantages over existing methods [22-36] with respect to sensitivity, selectivity, range of determination, simplicity, speed, pH/acidity range, thermal stability, accuracy, precision and ease of operation. Oxidation is very swift and no extraction is required throughout the entire process. With appropriate masking agents, the reaction can be compiled to be immensely selective and the reagent blank solutions hardly show any fluorescence.

Experimental Section

Apparatus

A Shimadzu Spectrofluorophotometer (Kyoto, Japan, Model: RF-5301PC), with 1 cm quartz cells were used and a Jenway pH meter (England, UK, Model: 3010) with combination of electrodes were employed for overall measurements of the fluorescence intensity and the pH, respectively. The calibration and linearity of the instruments were frequently monitored with standard quinine sulfate (10 μgL^{-1}). A Shimadzu double-beam UV/Vis Spectrophotometer (Kyoto, Japan, Model: 1800) was used to compare of the results. Infrared spectrum was recorded with FTIR Spectrophotometer, Shimadzu (Model: IR Prestige 21, Detector: DTGS KBr) in the range 7500 – 350 cm^{-1}. MLW type thermostatically controlled water bath (Memmert GmbH, Co. Schwabach, Germany) was used.

Reagents and solutions

All the chemicals used were of analytical reagent grade of the highest purity available. High-purity doubly distilled de-ionized water was used throughout the entire experiment. High-purity water was obtained by passing tap water through cellulose absorbent and to mixed-bed ion exchange columns, followed by distillation in a corning AG-11 unit. Glass vessel were cleaned by soaking in acidified solutions of $KMnO_4$ or $K_2Cr_2O_7$ followed by washing with concentrated HNO_3 and rinsed several times with high purity de-ionized water. Stock solutions and environmental water sample (1000 mL each) were kept in polypropylene bottles containing 1 mL concentrated HNO_3. More rigorous contamination control was used when the paracetamol levels in the specimens were low.

Paracetamol stock solution (6.62×10^{-3} M)

A 100 mL amount of stock solution (1-$mgmL^{-1}$) of PR was prepared by dissolving the requisite amount (151.2 mg) of PR (Sigma-Aldrich, Merck, KGaA, Germany, pro-analysis grade, 99.7 %)) in a known volume of de-ionized water. More dilute solutions of the PR were prepared as and when required. A freshly prepared reagent PR solution (10 mgL^{-1}) was used whenever as required. The purity of PR was tested by taking the melting point, FTIR spectrum, elemental analysis and thermogravimetric analysis. The melting point of the paracetamol was found 169 ± 2°C (lit. 168°C) [51] and the elemental analysis data was found as C = 63.0 %, O = 21.15 %, N = 9.12 % and H = 5.58 % (lit. C = 63.57 %, H = 6.00 %, N = 9.27 % and O = 21.17 %) [52]. The FTIR spectrum of the reagent (paracetamol) is as exhibited in Fig. 1. The appearance of FTIR peak at 1656.82 cm^{-1} in Fig. 1 was due to the innate C=O double bond peak (lit. $\nu_{C=O}$ = 1640 – 1690 cm^{-1}) [53] and the peak at 3326.39 cm^{-1} in Fig. 1 was due to the innate O-H bond peak (lit. ν_{O-H} = 3200 – 3600 cm^{-1}) [53] of the reagent indicating the presence of PR. The thermogravimetric curve of the reagent PR is shown in Fig. 2. Both the melting point, elemental analysis and FTIR spectral analysis data reported the purity of PR. The firmness of the thermogravimetric curve attained from about 1 g of the reagent at 80 – 90°C, which indicates that the reagent did not contain any moisture.

The elemental analysis was accomplished by the National Center of Excellence in Analytical Chemistry, University of Sindh, Pakistan and the FTIR spectra was recorded in the range 7500 – 350 cm^{-1} in our laboratory.

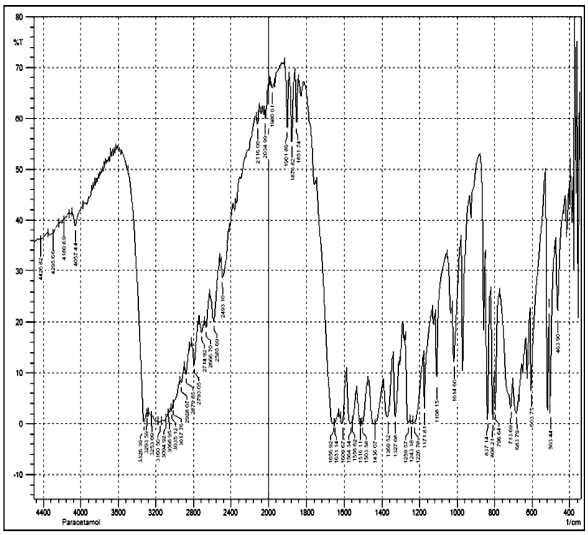


Figure1. **FTIR spectrum of *N*-acetyl-4-aminophenol (paracetamol)**

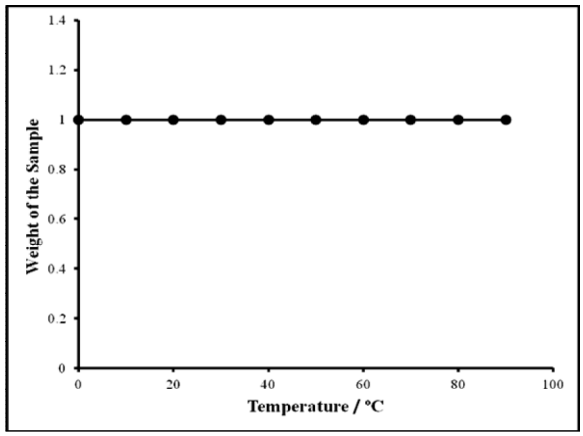


Figure 2. **Thermogravimetric curve of *N*-acetyl-4-aminophenol (paracetamol) at 80 - 90°C.**

Cerium (IV) standard solution (7.14×10^{-3} M)

A 100 mL amount of stock solution (1 $mgmL^{-1}$) of tetravalent cerium was prepared by dissolving 288.5-mg of ceric sulfate tetra-hydrate {$Ce(SO_4)_2.4H_2O$} (Sigma-Aldrich, Merck KGaA,

Germany, pro-analysis grade, 99.6 %) in doubly distilled de-ionized water and standardized by titrimetry with ethylenediaminetetraacetic acid (EDTA) using *o*-phenanthroline solution (ferroin) as indicator [54]. More dilute standard solutions were prepared by appropriate dilution of aliquots from the stock solution with de-ionized water as and when required. A freshly standardized solution (10 mgL^{-1}) was always used.

Ammonium sulfate solution

Ammonium sulfate solution (2 % w/v) (A.C.S-grade 99 % pure) was freshly prepared by dissolving 2 g in 100 mL of de-ionized water.

Aqueous ammonia solution

A 100 mL solution of an aqueous ammonia solution was prepared by diluting 10 mL concentrated NH_4OH (28 – 30 %, A.C.S.-grade) to 100 mL with de-ionized water. The solution was stored in a polypropylene bottle.

Other solutions

Solutions of a large number of inorganic ions and complexing agents were prepared from their AnalaR grade or equivalent grade water-soluble salts (or the oxides and carbonates in hydrochloric acid); solutions of different drug samples were specially prepared (Specpure, Johnson Matthey) according to the recommended procedures of Mukharjee [55]. In the case of insoluble substances, special dissolution methods were adopted [56].

Procedure

To 0.1 - 1.0 mL of a neutral aqueous solution containing 100 – 7000 ng of *N*-acetyl-4-aminophenol (PR) in a 10 mL calibrated flask was mixed with 5 – 8 μgL^{-1} (preferably 0.6 mL of 7.14×10^{-7} M) of the cerium (IV) solution followed by the addition of 0.5 – 1.5 mL (preferably 1 mL) of 0.1 M of sulfuric acid. The reaction solutions were mixed well and then the mixture was diluted to the mark with de-ionized water and heated in a thermostatically controlled water bath at 45 ± 5 °C for 5 minutes. The flask was then allowed to cool at room temperature (25 ± 5°C) for 15 minutes, the fluorescence intensity of the system was measured at 350 nm against a corresponding reagent blank, prepared concurrently, keeping the excitation wavelength maximum at 255 nm and the instrument setting the same. The PR content in an unknown sample was determined using a concurrently prepared calibration graph.

Sample collection and preservation

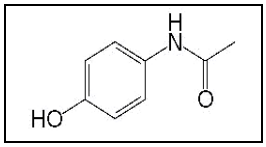
***Blood, urine and milk*:** Blood and urine samples were collected in polythene bottles from effected persons who have taken paracetamol for their relief from fever or sever pain of Chittagong Medical College Hospital, Bangladesh. Milk sample was collected from a Bangladeshi lactating mother who has taken paracetamol. Immediately after collection they were stored in a salt-ice mixture and latter, at the laboratory, were at -20 °C.

***Pharmaceutical samples*:** Pharmaceutical samples (tablet, suppository, drop and syrup) of different commercial companies were collected from local pharmacies of Chittagong. Samples (tablet and suppository) were homogenized with a mortar.

Results & Discussion

Factors affecting the fluorescence intensity
Spectral characteristics

The excitation and emission spectra of the fluorescent PR – Ce(IV) system in 0.1 M sulfuric acid medium was recorded using the spectrofluorophotometer. The excitation and emission maxima were at 255 nm and 350 nm, respectively. The reagent blank exhibited negligible fluorescence, despite having wavelength maximum in the same region. In all instances, measurements were made against the reagent blank. The fluorescence spectra are shown in Fig. 3. The structure of PR is shown in the Scheme-I.



Scheme-I. Structure of paracetamol (*N*-acetyl-4-aminophenol)

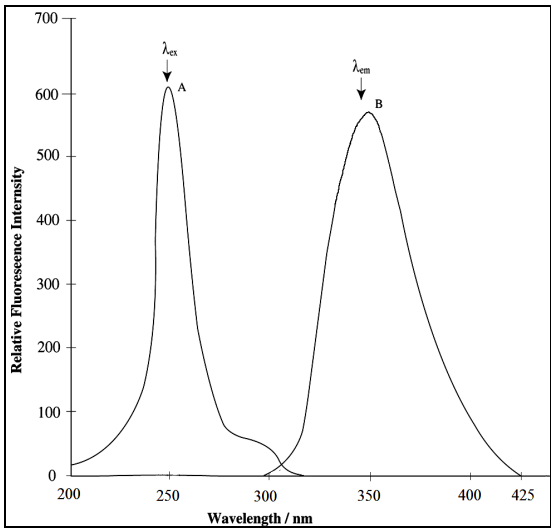


Figure 3. **Spectra A & B are the excitation and emission spectra of paracetamol - Ce^{IV} system (λ_{ex} = 255 nm, λ_{em} = 350 nm), respectively in aqueous solutions**

Optimization of some parameters on the fluorescence intensity

Effect of acidity

Among various acids (nitric, sulfuric, hydrochloric and phosphoric) studied, sulfuric acid was found to be the best acid for the system. Hence for the proposed procedure of the spectrofluorimetric determination of paracetamol with cerium, H_2SO_4 is better than other mineral acids. The fluorescence intensity was at maximum and constant when the 10 mL of solution (100 μgL^{-1} of PR) contained 0.5 – 1.5 mL of 0.1 M sulfuric acid at temperature 45 ± 5 °C. Outside this range of acidity, the fluorescence intensity decreased (Fig. 4). The optimum acidity range in the final solution is therefore 0.05 – 0.15 M of H_2SO_4. For all subsequent measurements 1 mL of 0.1 M sulfuric acid was added.

Effect of temperature

The PR–Ce(IV) system attained maximum and constant fluorescence intensity when the reaction was heated for 5 minutes at 40 – 80°C temperature and then cooled for 15 minutes (Fig. 5) at room temperature (25 ± 5°C). Hence all subsequent measurements the solution was heated for 5 minutes at 45 ± 5°C and then cooled for 15 minutes at room temperature.

Effect of time

The PR – Ce(IV) system attained maximum and constant fluorescence intensity was obtained just after the reaction mixture was heated for 5 minutes at 45 ± 5°C and then cooled for 15 minutes at room temperature (25 ± 5°C) and stayed rigorously unaltered for 24 h as shown in Fig. 6.

Effect of Ce(IV) concentration

The effect of Ce(IV) concentration was investigated using different concentrations of the reagent in 0.1–50 μgL^{-1} employing concentration of the studied paracetamol 500 μgL^{-1}. Maximum and constant relative fluorescence intensity obtained with a Ce(IV) concentration of 5–8 μgL^{-1} for PR (Fig. 7). Hence, a Ce(IV) concentration of 6 μgL^{-1} was selected and all subsequent measurements were done at this concentration.

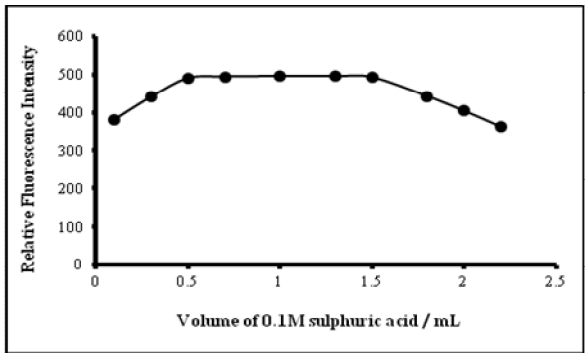


Figure 4. **Effect of acidity on the fluorescence of paracetamol – Ce(IV) system**

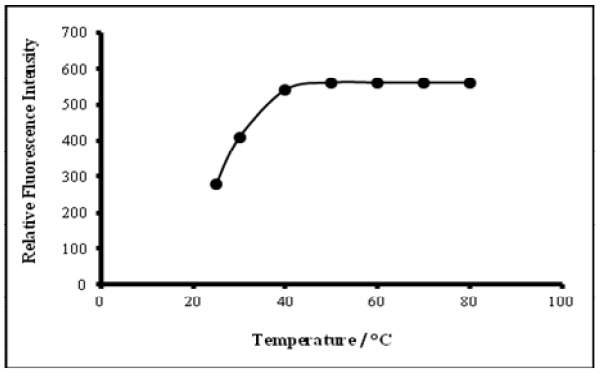


Figure 5. **Effect of temperature on the fluorescence of paracetamol – Ce(IV) system**

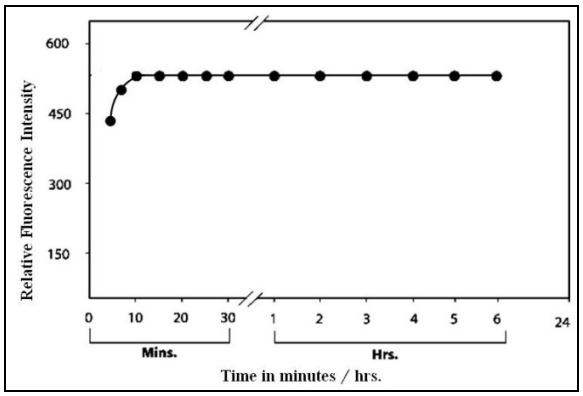


Figure 6. **Effect of the time on the fluorescence of paracetamol - Ce^{IV} system**

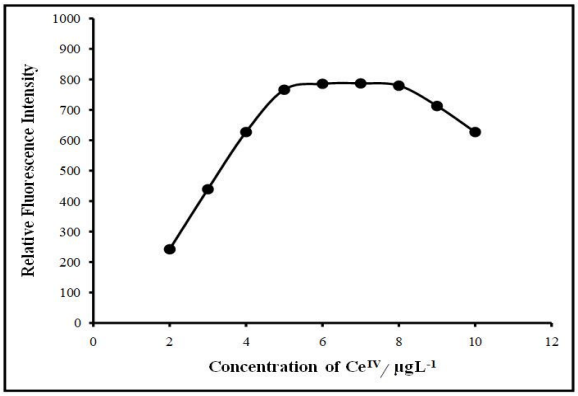


Figure 7. **Effect of Ce(IV) concentration on the fluorescence intensity induced due to oxidation of 100 µgL^{-1} for paracetamol on the paracetamol – Ce(IV) system**

Effect of paracetamol concentration

The well-known equation for spectrofluorimetric analysis in very dilute solutions derived from Beer's law. The effect of PR concentration was studied over 0.1 - 1000 µgL^{-1} distributed in four different sets (0.1 - 1, 1 - 10, 10-100 and 100-1000 µgL^{-1}) for convenience of measurement. The fluorescence intensity was linear over a wide range [10 ngmL^{-1} to 700 ngmL^{-1} or 10 – 700 µgL^{-1}] of PR at excitation wavelength at 255 nm and emission wavelength at 350 nm representing two linear graphs (10 – 100 and 100 – 700 µgL^{-1}) as shown in (Fig. 8 and Fig. 9), respectively. Among two calibration graphs, one exhibits the limit of the linearity range (Fig. 9); the other one (Fig. 8) shows a straight-line graph going through the origin (R^2 = 0.9999). The limit of detection and limit of quantification were found to be 2 µgL^{-1} and 10 µgL^{-1}, respectively.

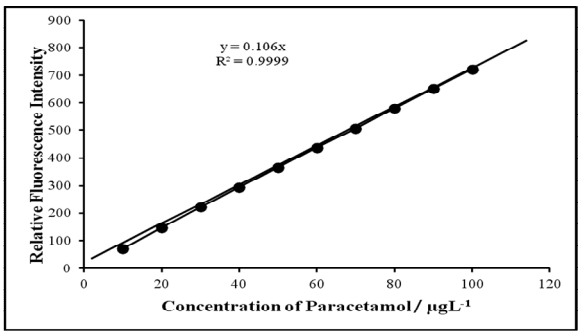


Figure 8. **Calibration graph A: 10 - 100 µgL^{-1} of paracetamol**
Bandwidth: Ex. slit - 5, Em. slit – 10
Sensitivity: High

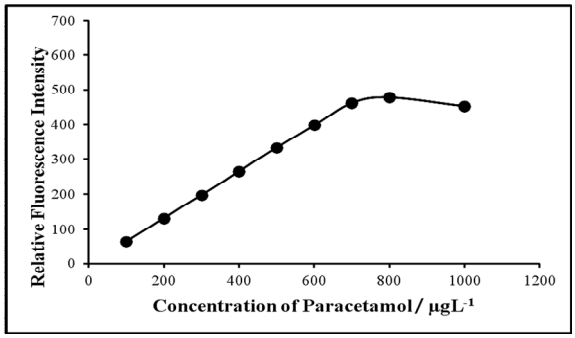


Figure 9. **Calibration graph B: 100 – 700 µgL^{-1} of paracetamol**
Bandwidth: Ex. slit - 10, Em. slit - 5
Sensitivity: High

Determination of molar ratio

The Job's method of continuous variation [57] was employed. Master equimolar solutions (3×10^{-6} M) of the investigated antiviral drugs and Ce(IV) reagent were prepared [57]. These solutions were prepared in 0.1 M sulphuric acid for PR and Ce(IV). Series of 10 mL portions of the master solutions of the PR and the reagent were made up comprising different complimentary ratios (0:10, 1:9, 9:1, 10:0, inclusive) in 10 mL volumetric flasks. The reactions were allowed to proceed under optimum conditions cited under the general assay procedure [57]. The fluorescence intensity of the resulting solutions were measured at λ_{ex} = 255 nm and λ_{em} = 350 nm against reagent blanks treated similarly. The stoichiometry of the reactions was assessed by Job's method [58] and the results proved that the drug/reagent ratio was 1:2 as shown in (Fig. 10). The selected analytical parameters obtained with the optimization experiments are summarized in Table 1.

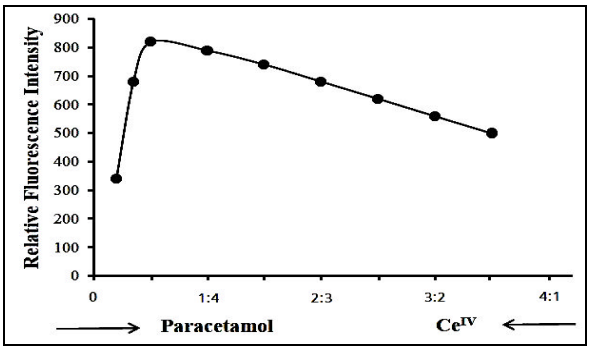


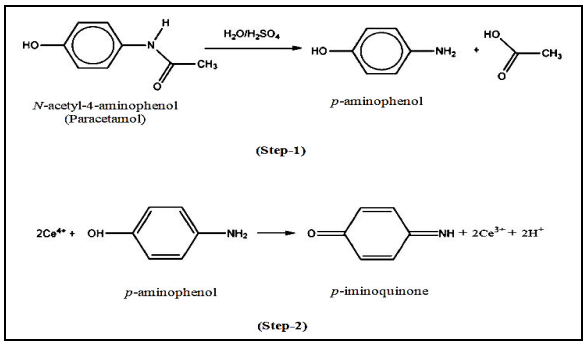
Figure 10. **Job's method for determining composition of paracetamol – Ce(IV) system in aqueous solutions (paracetamol: Ce(IV) = 1: 2)**

Table 1. **Selected analytical parameters obtained with the optimization experiments.**

Parameters	Studied range	Selected value
Excitation wavelength maximum / λ_{ex} (nm)	200 - 700	255
Emission wavelength maximum / λ_{em} (nm)	200 - 700	350
Acidity / M H_2SO_4	0.001 - 1.0	0.05 - 0.15 (Preferably 0.1)
pH	2.37 - 0.08	1.03 - 0.26 (Preferably 0.64)
Time / h	0 - 72	1min - 24 h (Preferably 15 min)
Temperature / °C	10 - 90	40 - 80 (Preferably 45 ± 5)
Reagent (Cerium) / μgL^{-1}	0.1 - 50	5.0 – 8.0 (Preferably 6.0)
Linear range / μgL^{-1}	0.1 - 1000	10- 700
Limit of quantification / μgL^{-1}	1 - 100	10.0
Detection limit / μgL^{-1}	1.0 - 10.0	2.0
Reproducibility (% RSD)	0 - 10	0 - 2
Regression Co-efficient (R^2)	0.9999	0.9999
Mole Ratio (drug : reagent)	0:10 – 10:0	1:2

Nature of the fluorescent species

The non-fluorescent species, PR, produces the same spectral characteristics with excitation and emission wavelengths almost invariably at 255 nm and 350 nm, respectively with cerium(IV), chromium(VI), Fe(III), Mn(VII) and with persulfate in acidic media. This implies that the fluorescent species is an oxidized product of the PR itself and not a chelate. Similar oxidative fluorescent reactions have been utilized previously [22]. The method was founded on the oxidation reaction of PR with Ce(IV) in sulfuric acid media. At higher concentrations of sulfuric acid the redox potential of cerium(IV) is such that it can be oxidized. This indicates that de-acetylation of PR to *p*-aminophenol (Scheme-II, Step-1) is the rate-determining step. *p*-aminophenol is then further oxidized with Ce(IV) to *p*-iminoquinone (Scheme-II, Step-2) [22].



Scheme-II. **De-acetylation of paracetamol to *p*-aminophenol**

Effect of foreign ions

About 40 drugs, pharmaceutical formulations and ions were studied individually which are commonly found in formulations and/or biological fluids to investigate their effects on the determination of 50 μgL^{-1} of PR. The criterion for interference was a fluorescence intensity value varying by more than ± 5 % from the expected value for PR alone [59]. The results are summarized in Table 2. A 500 μgL^{-1} level of each potentially interfering species was tested first, and, if interference occurred, the ratio was reduced progressively until interference ceased. As can be seen a large number of drugs, pharmaceuticals and ions have no significant effect on the determination of PR. The most serious interference was from salicylic acid and acetyl salicylic acid. Interferences from salicylic acid and acetyl salicylic acid were easily removed. The greater tolerance limits for these ions can be achieved by previous extraction of interfering species. The sources of interference species can be removed by previous extraction of those species with ethyl ether as indicated in the procedure [23]. A 200 and 300 fold of salicylic acid and acetyl salicylic acid, respectively, can be extracted with ethyl ether. For this purpose, the sample solution (5 mL) was 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 minutes as recommended by Vilchez *et al.* [23]. During the interference studies, if a precipitate was formed, it was removed by centrifugation. The amount mentioned is not the tolerance limit but the actual amount studied. However, for those ions whose tolerance limits have been studied, their tolerance ratios are mentioned in Table 2.

Table 2. **Table of tolerance limits of foreign ions[a], tolerance ratio [species(x) / parcetamol (w/w)].**

Species x	Tolerance ratio x/paracetamol (w/w)	Species x	Tolerance ratio x/paracetamol (w/w)
Acetamide	500	Galactose	500
Acetyl salicylic acid	300[b]	Glucose	500
Aminophenazone	700	Lactose	500
Ammonium	500	L-lysin	500
Aniline	1000	Maltose	400
Arabic gum	1000	Mandelic acid	500
Ascorbic acid	1000	Mannitol	400
Azide	1000	Morphine HCl	1000
α-cyclodextrin	600	Oxalate	500
Caffeine	800	*p*-aminophenol	700
Calcium gluconate	500	Phenacetin	600
Calcium lactobionate	500	Procaine HCl	1000
Carbutamide	1000	Propyphenazone	500
Cellulose	500	Saccharose	800
Citrate	1000	Salicylic acid	200[b]
Codeine	800	Sorbitol	500
Dextrin	1000	Starch	700
EDTA	1000	Sucrose	800
Ethyl cellulose	500	Thioridazine	1000
Fructose	1000	Thiourea	500

[a]Tolerance limit was defined as ratio that causes less than ± 5 percent interference.
[b] With 10 mgL^{-1} ethyl ether.

Precision and accuracy

The present method was justified according to FDA guidelines [60]. The precision of the present method was evaluated by determining various concentrations of PR (each analyzed at least five times). The relative standard deviation (n = 5) was 2 – 0 % for 100 – 7000 ng of PR in 10 mL, indicating that this method is highly precise and reproducible (Table 1). The detection limit (3s/S,'s' is the standard deviation of the blank & 'S' is slope) and limit of quantification (10 times of detection limit) for PR were found to be 2 μgL^{-1} and 10 μgL^{-1}, respectively. The method was also tested by analyzing several synthetic mixtures containing PR and diverse ions (Table 3). The reliability of the procedure was tested by recovery studies. The average percentage recovery obtained for addition of PR to few synthetic mixtures was quantitative, as shown in Table 3. The results of the synthetic mixture analyses were found to have an excellent recovery as compared to that obtained by spectrophotometry. The results of pharmaceutical analyses by the spectrofluorimetric method were found to have a profound recovery (Table 4). Also the results of biological analyses by the spectrofluorimetric method were in excellent agreement with those obtained by spectrophotometer. The results of biological analyses are shown in Table 5. Hence, the precision and accuracy of the method were found to be excellent.

Recovery study of paracetamol in synthetic mixtures

To inspect the accuracy of our proposed method, a recovery study was performed on a few synthetic mixtures. For this, numerous synthetic mixtures of sundry compositions comprised of PR and isolated pharmaceuticals of known concentrations were determined by the present method. The results were obtained to be highly reproducible. Unerring recoveries were achieved in all solutions. The reliability of our procedure was approved by quantitative recovery of PR spiked in several synthetic mixtures containing PR and diverse pharmaceuticals. The results of recoveries (99.94 ± 1.8 to 100.0 ± 0.0) of synthetic mixtures by the spectrofluorimetric method were also found to be in fantastic agreement with those obtained by the spectrophotometric method. The method exhibits high precision and accuracy (s = ± 0.01 for 50-μgL^{-1}). Table 3 shows the results obtained by the proposed method.

Table 3. **Recovery study of paracetamol in synthetic mixtures.**

Sample	Composition of Mixtures (μgL^{-1})	Paracetamol (μgL^{-1})				
		Added	Proposed Method (n = 5)		Spectrophotometry (n = 5)	
			Found[a]	*Recovery ± SD*[b] *(%)*	*Found*[a]	*Recovery ± SD*[b] *(%)*
A	Paracetamol	10	10.00	100.0 ± 0.0	9.99	99.9 ± 1.0
		50	49.98	99.96 ± 1.0	49.97	99.94 ± 1.5
B	As in A + L-lysin (50) + Thiourea (50) + Dextrin (50) + Codeine (50) + Arabic gum (50) + Ascorbic acid (50)	10	10.00	100.0 ± 0.0	9.98	99.8 ± 1.6
		50	49.98	99.96 ± 1.4	49.96	99.92 ± 1.6
C	As in B + Mannitol (50) + Phenacetin (50) + Maltose (50) + Sucrose (50) + Cellulose (50)	10	9.98	99.8 ± 1.2	9.97	99.7 ± 1.7
		50	49.97	99.94 ± 1.8	49.95	99.9 ± 2.0
D	As in C + Aniline (50) + Aminophenazone (50) + *p*-aminophenol (50) + Phenacetin (50) + Galactose (50) + Saccharose (50)	10	9.96	99.6 ± 1.9	9.95	99.5 ± 2.2
		50	49.95	99.9 ± 1.8	49.93	99.86 ± 2.5
E	As in D + Lactose (50) + Sorbitol (50) + Glucose (50) + Caffeine (50) + Thioridazine (50)	10	9.95	99.5 ± 2.0	9.94	99.4 ± 2.6
		50	49.90	99.8 ± 1.8	49.89	99.78 ± 2.0

a Average of five analyses of each sample. b The measure of precision is the standard deviation (SD).

Determination of paracetamol in pharmaceutical formulations

Soluble solid samples and liquid samples were purchased from local markets and pharmacies. Pharmaceutical samples (tablet and suppository) were grinded to a fine powder in a mortar, blended and homogenized, and finally sieved through a 0.1 mm pore diameter plastic sieve. All samples were kept in clean dry containers. Solid soluble samples (5 – 30 mg) were directly weighed into the glass minicolumn (dissolution cell). Then, each minicolumn was assembled to the continuous ultrasound-assisted dissolution system, and immersed into the ultrasonic bath [61]. After, the dissolution circuit was loaded with the dissolving solution (1 mL of de-ionized water). Twenty tablets or 10 suppositories were weighed, finely powdered and 50 mL PR containing syrup or drop. An accurately weighed quantity of the powdered tablet or suppositories or syrup or drop stuffs equiponderant to 100 mg of the operative components was shifted to a 100 mL calibrated flask, dissolved in about 40 mL of distilled water (for powdered tablet, suppositories), or in 0.1 N sulphuric acid (syrup or drops) following a method recommended by Soysa *et al.* [62]. The stuffs of the flask were swirled, sonicated for 5 minutes, and then uplifted to the volume with de-ionized water. The mixtures were compounded well and filtered and the first portion of the filtrate was discarded. A measured volume (2.5 mL) of the filtrate was shifted to a 25 mL calibrated flask, and diluted quantitatively with de-ionized water to yield a working standard solution containing 10 μgmL^{-1}. An aliquot (1 – 2 mL) of this digested sample was taken into a 10 mL calibrated flask and then the PR content was determined as described under the general Procedure. The results of some pharmaceutical analyses are in excellent agreement with the reported (claimed) values. The results of pharmaceutical analyses by the spectrofluorimtric method were found to be in fantastic agreement with those obtained by British Pharmacopoeia Method [63]. The recovery percentages ranged from 90.4 ± 1.5 to 106.2 ± 2.2 % (Table 4). These results were analogous with those gained from the Official method [62] by statistical analysis with respect to the accuracy (t-test) and precision (F-test) [64]. No serious incongruence was found between the calculated and theoretical values of both the proposed and the reported method at 95 % confidence level. This implies similar accuracy and precision in the analysis of the investigated compounds in their pharmaceutical dosage forms [65]. The results of several Pharmaceutical Companies for PR are given in Table 4. Very low values for PR in the some of the samples were probably due to inaccurate formula or techniques of preparations in those commercial companies.

Table 4. **Determination of paracetamol (PR) in some drug formulations.**

No.	Brand Name	Sample Type[a]	Composition of Sample	Trade Name	Paracetamol ($mgkg^{-1}$ or mgL^{-1})			RSD[b] (%)	British Pharmaco-poeia Method
					Reported Value (mg)	Found Paracetamol (n = 5)	Recovery (%)		
1	Beximco Pharmaceuticals Ltd. (BPL)	Tablet	PR: 500 mg	Napa	500.0	505.0	101.0± 0.5	1.0	100.2 ± 0.3
			PR: 500 mg, Caffeine: 65 mg	Napa Xtra	500.0	499.0	99.8 ± 1.5	2.0	-
			PR: 665 mg	Napa Extend	665.0	666.0	100.1± 1.8	2.1	-
		Syrup	PR: 24 mg	Napa	24.0	25.5	106.2± 1.5	2.2	-
		Supp[c]	PR: 125 mg	Napa	125.0	124.5	99.6 ± 1.0	2.5	99.8 ± 0.5
2	Square Pharmaceuticals Ltd.	Tablet	PR: 500 mg	Ace	500.0	498.8	99.8 ± 1.5	2.5	100.2 ± 0.3
			PR: 500 mg Caffeine: 65 mg	Ace Plus	500.0	499.5	99.9 ± 1.0	2.0	-
			PR: 665 mg	Ace XR	665.0	670.0	100.7± 1.5	1.6	-
		Syrup	PR: 24 mg	Ace	24.0	23.8	99.2 ± 1.0	2.2	
		Supp[c]	PR: 125 mg	Ace	125.0	130.0	104.0± 1.5	2.5	99.8 ± 0.5
3	Incepta Pharmaceuticals Ltd.	Tablet	PR: 500 mg	Renova	500.0	500.0	100.0± 0.0	0.0	100.2 ± 0.3
			PR: 500 mg Caffeine: 65 mg	Renova Plus	500.0	495.5	99.1 ± 1.5	2.5	-
			PR: 665 mg	Renova XR	665.0	668.0	100.4± 1.8	2.0	-
		Syrup	PR: 24 mg	Renova	24.0	25.8	107.5± 1.5	2.0	-
		Supp[c]	PR: 125 mg	Renova	125.0	126.0	100.8± 1.2	1.8	99.8 ± 0.5
4	Eskayef Pharmaceuticals Ltd.	Tablet	PR: 500 mg	Tamen	500.0	503.0	100.6± 1.5	1.6	100.2± 0.3
			PR: 500 mg Caffeine: 65 mg	Tamen Turbo	500.0	495.0	99.0 ± 1.0	3.0	-
			PR: 665 mg	Tamen XR	665.0	662.0	99.5 ± 2.0	2.5	-
		Syrup	PR: 24 mg	Tamen	24.0	23.9	99.6 ± 1.5	2.0	-
5	Aristopharma Ltd.	Tablet	PR: 500 mg	Xpa	500.0	505.0	101.0± 1.6	2.8	100.2± 0.3
			PR: 500 mg Caffeine: 65 mg	Xpa C	500.0	498.5	99.7 ± 1.5	2.0	-
			PR: 665 mg	Xpa XR	665.0	668.0	100.4± 1.0	2.6	-
		Syrup	PR: 24 mg	Xpa	24.0	25.0	104.2± 2.0	2.8	-
6	The ACME Laboratories Ltd.	Tablet	PR: 500 mg	Fast	500.0	497.8	99.6 ± 2.0	2.5	100.2± 0.3
			PR: 500 mg Caffeine: 65 mg	Fast Plus	500.0	498.0	99.6 ± 1.5	2.0	-
			PR: 665 mg	Fast XR	665.0	669.0	100.6± 1.8	2.6	-
		Syrup	PR: 24 mg	Fast	24.0	25.0	104.2± 1.0	2.0	-
7	Glaxo SmithKline	Tablet	PR: 500 mg	Parapyrol	500.0	500.0	100.0± 0.0	0.0	100.2± 0.3
		Syrup	PR: 24 mg	Parapyrol	24.0	25.6	106.7± 1.2	2.5	-
8	Jayson Pharmaceticals Ltd.	Tablet	PR: 500 mg	Zerin	500.0	450.4	90.8 ± 2.0	2.8	100.2± 0.3
		Syrup	PR: 24 mg	Zerin	24.0	22.4	93.3 ± 1.8	3.0	-
		Drop	PR: 16 mg	Zerin	16.0	14.9	93.1 ± 1.5	2.5	-
9	Zenith Pharmaceuticals Ltd.	Tablet	PR: 500 mg	Acep	500.0	467.8	93.6 ± 2.0	2.6	100.2± 0.3
			PR: 500 mg Caffeine: 65 mg	Acep Plus	665.0	621.5	93.4 ± 2.5	3.0	-
		Syrup	PR: 24 mg	Acep	24.0	21.7	90.4 ± 1.5	3.0	-

[a]Samples were collected from local market of Chittagong. [b]The precision is the relative standard deviation (RSD). [c]Suppository

Determination of paracetamol in biological fluids

Human blood and urine samples were obtained from healthy persons, patients suffering from acute fever and severe pain who had received a single oral dose of PR (665 mg in 100 mL). Blood samples were taken aseptically from an in-dwelling canola, pre-dose and at intervals for up to 8 h post-dose. Milk sample was collected from a Bangladeshi lactating mother who has taken paracetamol (665 mg). The protocol was approved by Ethics Committee of the Faculty of Medicine, University of Chittagong and the subjects gave their informed consent. Human blood or milk (1 – 2 mL) or urine (5 – 10 mL) sample was taken into a 100 mL micro-Kjeldahl flask. The sample was digested using solid-phase extraction methods suggested by Knepil [66]. The proteins of the samples were precipitating by adding ethanol and ammonium sulfate and shaking with chloroform. The organic extract was then evaporated, the residue dissolved in ether, and then back-extracted into 0.4 % sodium bicarbonate solution, continuing according to Routh *et al.* [67]. The content of the flask was heated after addition of 2 mL of 0.1 M H_2SO_4 for 0.5 h at 60°C to dissolve content. When initial brisk reaction was completed, the solution was removed and cooled at room temperature. Obtained solution was then filtered through a Whatman No. 40 filter paper and quantitatively shifted into a 25 mL calibrated flask followed by making up to the mark with de-ionized water. A suitable aliquot (1 – 2 mL) of the final solution was pipetted out into a 10-mL calibrated flask and the PR content was determined as described under the general procedure. The results of biological analyses by the spectrofluorimetric method were found to be in an excellent agreement with those obtained by spectrophotometry. The results are shown in Table 5.

The present method was statistically compared with some of the reported methods [22, 25, 26, 31, 32, 36]. It was found that present method is much superior those of the reported methods. The results are enlisted in Table 6.

Table 5. **Determination of paracetamol in some biological fluids.**

No.	Sample	Paracetamol (μgL^{-1})				Sample Source[a]
		Spectrophotometry (n = 5)		Proposed Method (n = 5)		
		Found	*RSD[b](%)*	*Found*	*RSD[b](%)*	
1	Blood	145.0	2.0	152.5	1.8	Kidney disease patient
	Urine	24.5	1.8	25.8	1.5	(Male)
2	Blood	134.8	2.2	135.5	2.0	Hypertension patient
	Urine	20.8	1.5	21.9	1.6	(Female)
3	Blood	124.6	2.5	125.8	2.0	Lung cancer patient
	Urine	18.9	1.8	20.5	1.7	(Female)
4	Blood	138.4	2.8	140.6	2.2	Liver cirrhosis patient (Male)
	Urine	21.5	1.8	22.8	1.8	
5	Blood	125.0	2.6	128.7	2.5	Diabetic patient
	Urine	19.6	1.5	20.8	1.6	(Female)
6	Blood	123.0	2.2	125.5	2.8	Smoker (Male)
	Urine	17.8	1.6	18.9	1.5	
7	Blood	133.5	2.0	134.7	2.1	Normal adult
	Urine	23.0	1.8	24.5	2.0	(Female)
8	Blood	90.5	1.6	95.0	1.8	Normal adult (Male)
	Urine	15.0	1.2	16.5	1.4	Non-smoker
9	Milk	86.0	1.2	88.5	1.5	Bangladeshi lactating mother

[a]Samples were collected from Chittagong Medical College Hospital
[b]The precision is the relative standard deviation (RSD)

Table 6. **Statistical comparison of proposed method with reference methods.**

Samples	F – test results [a]					
	(s_1^2/s_2^2) [22]	(s_1^2/s_3^2) [25]	(s_1^2/s_4^2) [26]	(s_1^2/s_5^2) [31]	(s_1^2/s_6^2) [32]	(s_1^2/s_7^2) [36]
Blood						0.48
Synthetic mixture				0.167	0.1498	
Synthetic mixture				0.0356	0.2341	
Pharmaceutical formulation	0.783	0.041	0.1372	0.1936	0.1040	
Pharmaceutical formulation	0.1168					
Urine						0.36

[a]Tabulated F-value for (5,5) degrees of freedom at *P*(0.98) is 5.72. S_1=Standard deviation of proposed method, S_2= Standard deviation of reference method [22], S_3= Standard deviation of reference method [25], S_4= Standard deviation of reference method [26], S_5= Standard deviation of reference method [31], S_6= Standard deviation of reference method [32], S_7= Standard deviation of reference method [36]

Conclusion

A new swift, ultra sensitive, extremely selective and get-at-able spectrofluorimetric method with the paracetamol – Ce(IV) system was developed for the determination of PR in some synthetic mixtures, pharmaceuticals and biological fluids.

Although many sophisticated techniques such as pulse polarography, HPLC, AAS, ICP-OES, and ICP-MS, are available for the determination of PR at ultra-trace levels in numerous pharmaceutical and biological materials, factors such as the low cost of the instruments, easy handling, lack of requirement for consumables, and almost no maintenance have caused spectrofluorimetry to remain a popular technique, particularly in laboratories of developing countries with limited budgets.

The method is reliable for the accurate determination of these drugs in bulk and pharmaceutical dosage forms without interference from the common excipients. From the economical point of view, all the analytical reagents are inexpensive, have excellent shelf life, and are available in any analytical laboratory of developing countries. The sensitivity and precision in terms of relative standard deviation of the present method is very reliable for the determination of PR in real samples down to ngg^{-1} (10^{-9} gg^{-1}) levels in aqueous medium at room temperature (25 ± 5 °C). Therefore, this method can be successfully used in routine analysis of trace amounts of PR in biological fluids and pharmaceutical formulations. It is a new approach and could be an alternative method for the rapid determination of PR in a wide variety of sample solutions to the methods described in different literatures [22-36].

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