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Talanta 66 (2005) 229–235
Talanta
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Selective spectrofluorimetric method for paracetamol determination through coumarinic compound formation

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Received 20 May 2004; received in revised form 10 November 2004; accepted 18 November 2004
Available online 25 December 2004

Abstract

A spectrofluorimetrical selective method was designed for determination of paracetamol in tablets. This important technique can be characterized by its sensitivity, simplicity, celerity and cheaper cost than current official methods. The employed methodology involves coumarinic compound formation obtained by reaction between paracetamol and ethylacetoacetate (EAA) in the presence of sulphuric acid as catalyst. The reaction product is highly fluorescent at 478 nm, being excited at 446 nm.

The linear concentration range of the application was 0.1–0.4 μg/ml of paracetamol and the detection limit was 57 ng/ml.

The influence of different variables was studied and optimized through chemometric techniques. Applying the above-mentioned method good results were obtained with regard to pharmaceutical formulations containing paracetamol. Therefore, it is relevant to suggest this profitable technique for medicament control analysis.

Keywords: Fluorescence; Paracetamol; Ethylacetoacetate; Coumarin

1. Introduction

Paracetamol or acetaminophen (*N*-(4-hydroxyphenyl) acetamide) (Scheme 1) is an effective alternative to the aspirin as an analgesic and antipyretic agent. Compared to aspirin, paracetamol's anti-inflammatory activity is considered weaker.

This substance can be characterized by its tolerance, its gastric indisposition absence and its free selling. Furthermore, acetaminophen is often self-prescribed, without medical control, to alleviate moderate pain, to calm fever, lumbar pain, migraine or non-specific indications [1].

Recent studies have shown that paracetamol is associated to hepatic toxicity and renal failure despite of its apparent innocuous character. Hepatic toxicity begins with plasma levels of paracetamol in the 120 μg/ml range 4 h after the ingestion and an acute damage is presented with plasmatic levels up to 200 μg/ml 4 h after the ingestion.

At normal therapeutic doses, paracetamol is metabolized very fast and completely by undergoing glucuronidation and sulfation to inactive metabolites that are eliminated in the urine. However, paracetamol higher doses produce toxic metabolite accumulation that causes hepatocyte death.

Approximately 0.01% of the US population and 0.02% of the Australian population are assessed in hospital each year because of paracetamol poisoning [2].

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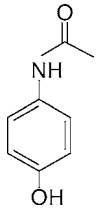
doi:10.1016/j.talanta.2004.11.015



Scheme 1.

Acetaminophen overdose is a frequent cause of fulminating hepatic failure in Europe and US [3].

Based on the aforementioned observations the main objective was to carry out the development of more efficient analytical techniques, destined to quality control of one of the medicaments more widely used.

Several analytical procedures are proposed for the determination of paracetamol in pharmaceutical products, e.g., titrimetric [4], colorimetric [5], UV–visible absorption [6], voltammetric [7], flow-injection systems (FIA) with colorimetric detection [8,9], Fourier transform infrared spectrometry [10], HPLC [6,11], micellar liquid chromatography [12] and many others.

Both the BP [6] and USP [11] recommend an HPLC method for the determination of paracetamol in pharmaceutical formulations, which requires high sophistication and very expensive cost of equipment.

The determination of paracetamol tablets can be carried out by direct ultraviolet absorption spectrophotometry (monograph in BP [6]), however, when formulated with other UV-absorbing substances such as excipients or other drugs, where spectral overlap is possible, separative techniques are necessary. The notorious advantages of the proposed methodology are the reduction of analytical costs and a very interesting alternative to those labs, which do not have such sophisticated equipments as required to carry out official techniques.

Many spectrofluorimetric methods have been proposed for the determination of paracetamol as single or as a mixture with other drugs in pharmaceutical formulations, such as indirect determination using Ce(IV) as an oxidant agent [13], reaction with fluorescamine [14], reaction with dansyl chloride [15], 1-nitroso-2-naphthol [16], potassium hexacyanoferate (III) [17], oxidation with 2,2′-dihydroxy-5,5′-diacetyldiaminebiphenyl [18]. However, many of these methods show low selectivity and interference with other drugs and excipients can be observed. This problematic situation does not appear with the proposed method for spectrofluorimetrical determination of paracetamol presented to this paper.

In this work, it is intended to develop a selective technique for paracetamol determination in pharmaceutical formulations, which can also contain other drugs without interference between them.

This methodology is based on reaction between paracetamol and ethylacetoacetate in presence of a dehydrating agent such as sulfuric acid producing a coumarinic compound, which is spectrofluorimetrically measured [19–22].

The developed method was applied to the analysis of four different commercial pharmaceutical tablets obtaining good results compared to those acquired through official methods, which involved the use of expensive equipment and reagents in routine analysis.

Finally, the importance of medicaments control is to guarantee the safety and the trust in pharmaceutical formulations, which are of common using and non-prescripted. Therefore, it is very relevant to emphasize that they may cause death due to its toxicity and absence control.

2. Experimental

2.1. Apparatus

All fluorescence measurements were made on a Shimadzu RF-5301 PC spectrofluorophotometer with excitation and emission band pass of 5 nm using 1.0 cm quartz cells.

A Beckman DU 520 UV–visible spectrometer with quartz cells of 10 mm path length for absorptiometric measurements was used. NMR spectra were obtained using a Bruker AC-200 spectrometer.

2.2. Reagents

The ethylacetoacetate (Anedra) labeled to contain 98% (v/v) was prepared as 2% (v/v) solution in absolute ethanol and should be freshly prepared. Sulfuric acid (Merck) labeled to contain 98% (v/v). Paracetamol was supplied by Novartis Lab, Argentina. All solvents used were HPLC grade and all other reagents employed were of analytical grade and were used without further purifications.

2.3. Preparation of the standard solution

An accurate mass 15 mg of weighted paracetamol was transferred into 100 ml volumetric flask and dissolved in absolute ethanol to obtain a standard solution of 1×10^{-3} mol l^{-1}.

A working standard paracetamol solution obtained by dilution with the same solvent to give final concentrations of 1×10^{-5} mol l^{-1}. From this solution, a series of dilutions were prepared in absolute ethanol to obtain a range of concentrations 7.5×10^{-7} to 3×10^{-6} M. The standard solution was protected from light. The solutions were stable for at least 1 week, if they had been stored in a dark place.

2.4. Construction of the calibration graphs

A set of volumetric flask aliquot solutions of working standard paracetamol were quantitatively transferred to each

flask. To these solutions were added 170 μl of ethylacetoacetate (2%, v/v solution) and 1170 μl of sulfuric acid. These mixtures were heated under reflux (65 °C) for 30 min.

The fluorescence of each reaction mixture, after being cooled to room temperature, was excited at 446 nm and measured at 478 nm.

The validation was carried out, through spectrophotometric method (official method BP) [6] and standard addition method.

3. Procedure for commercial tablets

Ten tablets were weighted and powdered. An accurately weighted amount of powder equivalent to 15 mg drug was transferred into a 100 ml volumetric flask. The flask was filled to a predetermined volume using absolute ethanol and the mixture was agitated and filtered. The filtrate was completed to volume with the same solvent. The procedure was carried out with the above-mentioned technique and according to the variables optimization realized by the chemometric method. The validation was realized by the standard addition method and compared to the official method (spectrophotometric method) [6].

4. Results and discussions

The adequate derivatization of paracetamol enhances the weak native fluorescence of the same drug. The 4-methylcoumarin obtained by condensation of the drug with ethylacetoacetate (EAA) in presence of sulfuric acid as a dehydrating agent is a highly fluorescent compound. This fact is due to the increased inflexibility of the system, decreasing thus the loss of energy through rotational relaxation. In consequence, there is an enhancement of the quantum yield of fluorescence (Fig. 1) [23,24].

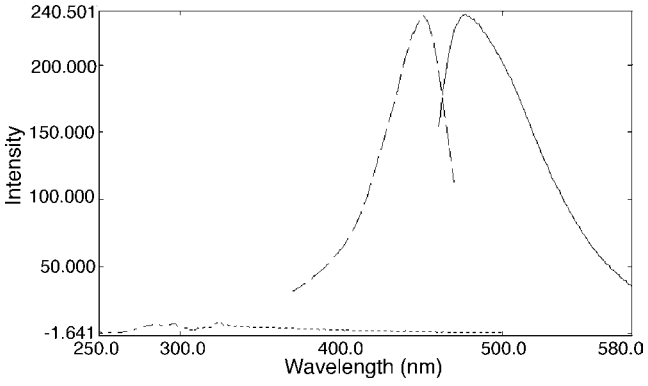
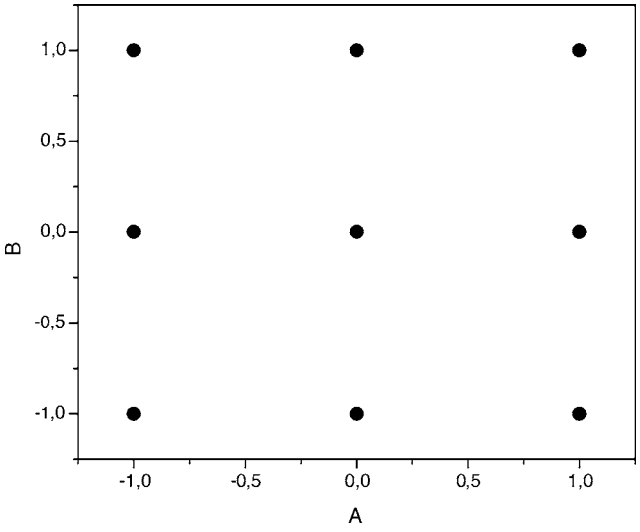


Fig. 1. Fluorescent excitation (– – –) and emission (- - - - - -) spectra of paracetamol (λ_{exc} = 274 nm, λ_{emi} = 306 nm), fluorescent excitation (— — —) and emission (—) spectra of the coumarinic compound produced by reaction between paracetamol and EAA (λ_{exc} = 446 nm, λ_{emi} = 478 nm).



Scheme 2.

5. Optimization of the reaction conditions

The experimental design proposes, by an efficient way, obtaining the optimization of a specific process or product with a minimum of experiments.

Experimental variables, such as temperature, reaction time, sulfuric acid volume and EAA concentration were optimized using experimental design (chemometric techniques).

These variables have a great influence in the fluorescence of the obtained product. The experimental design scheme used is shown in Scheme 2.

The matrix, which describes experimental design, is shown in Table 1.

In the first place, the temperature influence and reaction time on the relative intensity fluorescence of the reaction product was studied. The plot is shown in Fig. 2. It can be seen that the curve has a maximal response value, which corresponds to 65 °C temperature and 30 min reaction time. At higher temperatures to 80 °C, the fluorescent product is discomposed. In Fig. 3, it is possible to observe the influence of the EAA concentration and the acid volume on the fluorescence of the coumarin synthesis. It can also be verified that the highest fluorescence signal was observed when the reagent volume is equal to 170 μl and the acid volume is equal to 1170 μl. The obtained coumarin derivative is stable about 1 h.

6. Reaction mechanism

The Von Pechmann–Duisberg condensation is a prominent, efficient and simple reaction used to produce coumarins. Briefly, the procedure consists in condensation of phenolic derivated (paracetamol) with β-ketoesters in the presence of acid as catalyst, which is used in excess.

The mechanism is thought to involve the initial formation of a β-hydroxyester as above-mentioned [21].

Table 1
Matrix design of two factors and three levels (3^2)

Number of experiments	Experimental variables			
	A (temperature)[a]	B (time)[b]	C (volume of EAA)[c]	D (volume of sulphuric acid)[d]
1	−1	−1	−1	−1
2	0	−1	0	−1
3	1	−1	1	−1
4	−1	0	−1	0
5	0	0	0	0
6	1	0	1	0
7	−1	1	−1	1
8	0	1	0	1
9	1	1	1	1

[a] Temperature 20 °C (−1), 50 °C (0), 80 °C (1).
[b] Heating time 5 min (−1), 27 min (0), 60 min (1).
[c] Volume of EAA 0.5 ml (−1), 1.75 ml (0), 3 ml (1).
[d] Volume of sulphuric acid 0.3 ml (−1), 1.9 ml (0), 3.5 ml (1).

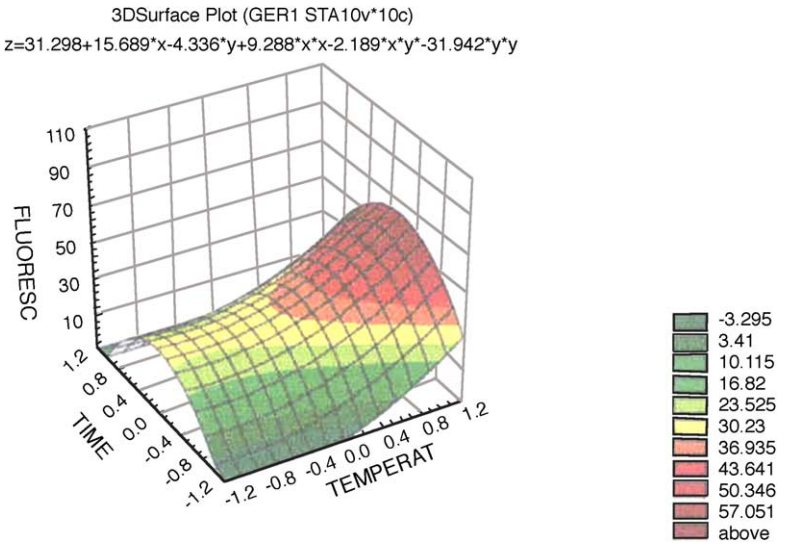


Fig. 2. Optimization of time and temperature for coumarinic product synthesis through experimental design.

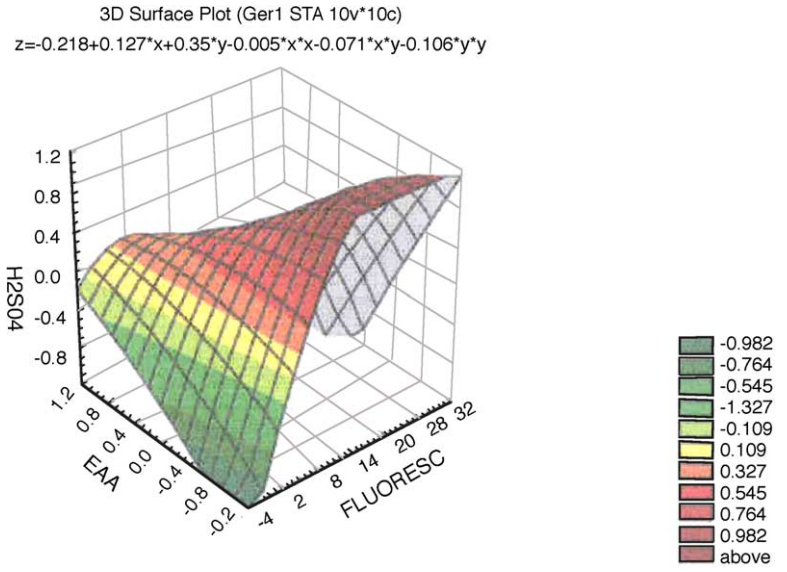
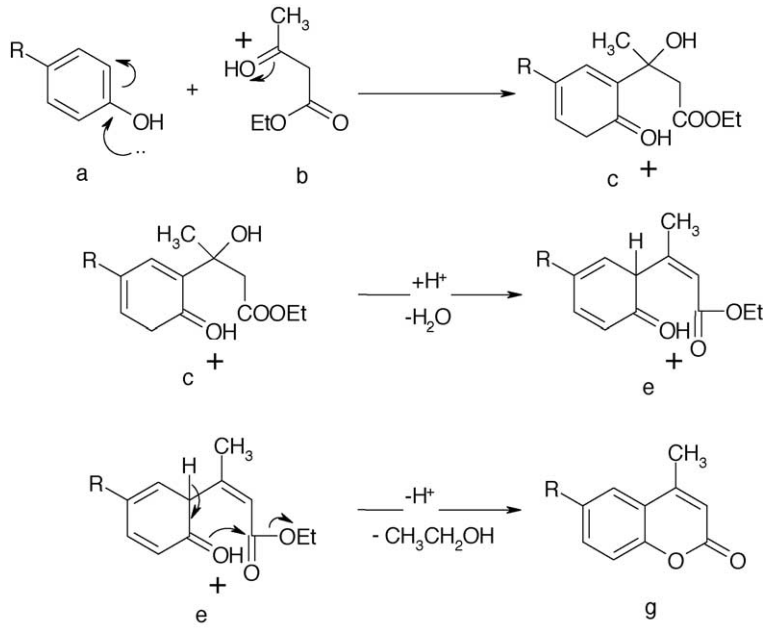


Fig. 3. Influence of sulfuric acid volume and EEA concentration in coumarinic product synthesis through experimental design.



Scheme 3. Proposal of the reaction pathway between paracetamol and EAA/sulphuric acid.

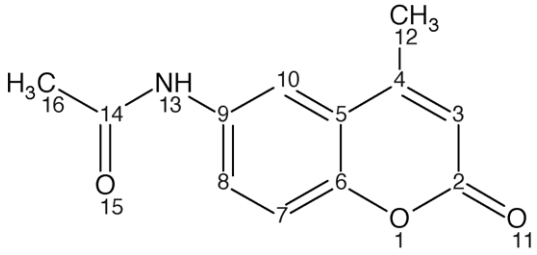
Ethylacetoacetate in presence of above-mentioned acid reacts very fast with paracetamol giving a yellow highly fluorescent product (Scheme 3).

The reaction mixture after being cooled to room temperature was poured onto crushed ice and stirred for 15–20 min. Then, this mixture was neutralized with sodium bicarbonate and then it has extracted the coumarine from reaction mixture twice with ethyl acetate. After extraction, the solvent was evaporated to dryness under reduced pressure and low temperature. The concentrated extract was used for spectroscopic study.

The compound was identified by comparing analytical data (^{1}H-NMR, ^{13}C-NMR, H–H COSY and capillary electrophoresis). The final product was a coumarinic derivate determinated through ^{1}H-NMR, ^{13}C-NMR, H–H COSY. The ^{1}H-NMR and ^{13}C-NMR data are shown in Table 2 (Scheme 4).

Table 2
^{1}H NMR and ^{13}C NMR spectral data for coumarinic product

Position	δ_C (ppm)	Position	δ_C (ppm)
12	21	H-12 (s)	1.7
16	23	H-16 (s)	2
3	112.5	H-3 (s)	5.9
10	116.5	H-7 (d)	7
8	121.2	H-8 (d)	7.5
5	121.3	H-10 (s)	7.65
7	121.7	H-14 (s)	8
9	135.2		
6	146		
4	153		
2	161		
14	168		



Scheme 4.

It was also verified by capillary electrophoresis that only one product was originated through Von Pechmann–Duisberg condensation. Only one peak can be observed at 480 nm that corresponds to coumarinic product.

7. Validation

Validation of the method was checked by the standard addition method (see Table 3) and also compared to the results with those obtained using an official method (BP) (Table 4) for the determination of paracetamol (spectrophotometric) [6].

The applied method shows goods results in order to quantify paracetamol in tablets independently of the excipients and the drugs contained in pharmaceutical formulations, e.g., caffeine, bromhexine, butethamate, diphenhydramine, astemizole, carbetapentane, chlorpheniramine, ascorbic acid, diclofenac, aminopyrine, piroxicam, ibuprofen and many others.

The selectivity of the method is based on the high reactivity EAA in the presence of paracetamol (phenolic

Table 3
Validation of proposed method by the standard addition method on a commercial sample

Sample	Quality of paracetamol added (mg)	Quality of paracetamol found in (mg) average ± S.D.
Tafirol®, (LASIFARMA), number = 6	0	503.6 ± 4.49
Paracetamol 500 mg of tablet	100	602.2 ± 5.51
Gripanil C®, (AGRAND/AHIMSA), number = 6	0	356.4 ± 11.2
Paracetamol 350 mg of tablet	100	459 ± 3.83
Qura®, (MICROSULES-BERNABO), number = 6	0	498.3 ± 3.76
Paracetamol 500 mg of tablet	100	598 ± 3.02
Dioxaflex Gesic®, (BAGÓ), number = 6	0	307.9 ± 6.02
Paracetamol 300 mg of tablet	100	398.1 ± 4.21

Table 4
Results for paracetamol-containing commercial tablets analyzed by two techniques

Sample number	Paracetamol found on commercial samples (mg)	
	Proposed method	Reference method
1	498.7	495.5
2	502.7	483.2
3	504	493.2
4	508.6	480.6
5	513.9	479.8
6	509.9	479.5
$X \pm$ S.D.	506.3 ± 5.51	485.3 ± 7.16

derivative) and sulphuric acid as catalyst to give a coumarinic ring as the final product. The drugs, which are present in pharmaceutical formulations and do not have phenolic structure, will not interfere with the proposed methodology.

The data were analyzed by linear regression least-square fit method. The calibration graph is described by the calibration equation $y = a + bx$, where y is the fluorescence intensity, b the slope, a the intercept and x the concentration of the coumarinic product.

Linear regression least-square fit data are given in Table 5.

The linear dependency of the drug concentration with relative fluorescent intensity was in the range between 0.1 and 0.4 μg/ml.

Detection limit (DL) is the lowest concentration that can be distinguished from the noise level. In this study, the DL was 57 ng ml^{-1}.

Table 5
The determined parameters for calibration curves of coumarinic product obtained from developed method

Parameter	$n = 5$
Linear dynamic range (μg ml^{-1})	0.1–0.4
Regression equation[a]	
Slope (b)	184.215
Error	8.52
Intercept (a)	63.631
Error	2.21
S.D.	1.98
LOQ (μg ml^{-1})	0.31
LOD (μg ml^{-1})	0.057

[a] $y = a + bx$, where x is the concentration in μg ml^{-1}.

Quantification limit (QL) is generally by the samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision. In this study, the QL was 0.31 μg ml^{-1}.

8. Analysis of commercial tablets

The method was applied for paracetamol determination in tablets of some commercially available formulations in Argentina, such as in single preparations or in mixtures with other drugs and compared to the official method. The concentrations of paracetamol were calculated by direct measurement using the appropriate calibration graph (Table 6).

Table 6
Paracetamol found on commercial samples using proposed method

Sample number	Paracetamol found on commercial samples (mg)			
	Tafirol®, (LASIFARMA), (paracetamol 500 mg of tablet)	Gripanil C®, (AGRAND/AHIMSA), (paracetamol (350 mg of tablet), ascorbic acid, caffeine)	Qura®, (MICROSULES-BERNABO), (paracetamol (500 mg of tablet), pseudoephedrine, bromhexine, astemizole)	Dioxaflex Gesic®, (BAGÓ), (paracetamol (300 mg of tablet), diclofenac)
1	498.7	348.6	495.7	302.5
2	502.7	352.3	501.9	316.9
3	504	358.4	489.2	308.3
4	508.6	360.2	496.3	298.5
5	513.9	360.4	502.9	307.2
6	509.9	358.5	503.8	314
$X \pm$ S.D.	506.3 ± 5.51	356.4 ± 4.83	498.3 ± 5.62	307.9 ± 6.87

The obtained results did not exhibit significant differences compared to the data obtained by the official method.

9. Conclusion

The fluorescence method proposed by under abovementioned technique can be considered as a precise, economical, rapid, selective and sensitive procedure for the determination of paracetamol in pharmaceutical formulations. This method does not require previous separation techniques due to no interference between paracetamol and excipients or other drugs, which are not capable to form the coumarinic product. Another advantage is the enhancement of fluorescent signal of the new product obtained and thus increased sensitivity.

Acknowledgements

The authors are grateful to National University of San Luis, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), and Agencia Nacional de Promoción Científica y Tecnológica for the financial support and Argentina Novartis Lab for kindly providing the drug.

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