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Development of the Second-order Derivative UV Spectrophotometric Method for Direct Determination of Paracetamol in Urine Intended for Biopharmaceutical Characterisation of Drug Products

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ABSTRACT: Paracetamol is a widely used nonsalicylate analgesic and antipyretic drug. The existing methods for the determination of paracetamol in biological fluids are mainly HPLC techniques, although there are some reported methods based on spectrophotometric determinations. However, all these methods involve some extraction or derivatisation procedures. In the present study the UV spectra of investigated samples were recorded over the wavelength range 220–400 nm (λ step 0.21 nm; scan speed 60 nm/min) and second-order derivative spectra were calculated. Second-order derivative spectra of different blank urine samples displayed the presence of a zero-crossing point at 245–247 nm defined as λ_{zc}. The zero-order absorption spectra of paracetamol in water displays maximum absorbance at 243 nm, while in second derivative spectra, a minimum peak at 246 nm was observed. Therefore, the application of zero-crossing technique to the second-derivative UV absorption spectrum should be useful for the determination of paracetamol using $^2D_{\lambda zc}$.

The proposed method enables determination of total paracetamol in urine directly and simply by reading the $^2D_{\lambda zc}$ of the diluted samples. The obtained results were in good accordance with published data on cumulative urinary excretion after per oral administration of paracetamol obtained applying different spectrophotometric methods of determination. It could be useful for biopharmaceutical characterisation of drug products (monitoring of the levels of paracetamol in urine in bioavailability testing, for the evaluation of *in vitro–in vivo* correlation and screening of different formulations during drug product development). Copyright © 2003 John Wiley & Sons, Ltd.

Key words: paracetamol; derivative UV spectrophotometry; determination in urine

Introduction

Paracetamol is used as analgesic and antipyretic drug, popular as an 'aspirin substitute' and available in different dosage forms from many sources. It is, also, useful in osteoarthritis therapy [1], and there is an increased interest for modified release dosage forms. Paracetamol is weak acid (pKa = 9.5) which is rapidly absorbed and distributed after oral administration and excreted largely in urine: 45%–55% as glucuronide conjugates, 20%–30% as sulphate, 15%–55% as cysteine and mercapturic acid conjugates and only 2%–5% unchanged [2]. Although paracetamol is a relatively safe analgesic, in high doses and over prolonged periods, its metabolism may

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produce a quantitatively minor metabolite of the drug that can destroy the enzyme systems responsible for its reduction.

The necessity for *in vivo* studies in the early stages of drug product development emphasises the need for a rapid and simple method for the determination of drug concentrations in biological fluids.

The existing methods for the determination of paracetamol in biological fluids (blood, plasma, urine) mainly use HPLC techniques [3–6], while there are some spectrophotometric methods in which paracetamol and its conjugates present in the urine are hydrolysed with acid to *p*-aminophenol which is coupled directly with phenol in the presence of hypobromite to form an indophenol dye [7] or with vanillin to form a yellow compound with the characteristic absorbance at 395 nm [8]. Damiani *et al.* [9] proposed the first derivative spectrophotometric method for the determination of paracetamol in blood serum. Another advantage of derivative spectroscopy was reported by Bermejo *et al.* [10] for the simultaneous identification and determination of plasma salicylate and paracetamol using second-order derivative spectra after a common extraction procedure.

Although, in many cases, it is considered that the best way to estimate pharmacokinetic data is by analysing drug blood levels, the use of a non-invasive method (e.g. urine samples) might allow the bioavailability characteristics of different preparations to be distinguished in a rapid and easy way. Measurement of the pharmacokinetic data of paracetamol formulations using urinary excretion data has been well documented [3, 7, 8, 11–14] and could be achieved without the discomfort, possible hazard and necessary attendance of medical staff required for repeated venipunctures. Recently, Criado *et al*. [15] reported a fully automated urinary screening system for paracetamol and its metabolites which comprises on-line acid microwave assisted hydrolysis of the drug to *p*-aminophenol followed by reaction with *o*-cresol in alkaline solution; the indophenol blue dye formed was monitored at 620 nm. Another optical technique, such as spectrofluorimetry, although more sensitive, also requires the use of derivative reactions [16] because paracetamol is not an intrinsic fluorophor. The determination would be more rapid and less expensive than that obtained by spectrofluorimetry if direct UV spectrophotometry were to be used.

This paper describes the use of second-order derivative UV spectrophotometry for direct, rapid and simple determination of paracetamol in urine without preliminary extraction or prior derivatisation procedures.

Materials and Methods

Reagents and solutions

Paracetamol was purchased from Merck (Schuchardt, Germany). Aqueous stock paracetamol solution (1 mg/ml) was used for preparation of standard solutions. Double-distilled water was used throughout. β-glucuronidase from *Helix pomatia* (Calbiochem, Darmstadt, Germany) was used for enzyme hydrolysis. For *in vivo* experiments paracetamol syrup (Paracet®, Zdravlje, Yugoslavia) was used. The urine samples, collected from healthy volunteers, were diluted 1:100.

Apparatus

UV absorption spectra were recorded on a GBC 914 spectrophotometer (GBC Scientific Equipment Pty Ltd, Dandenong, Australia). The instrument parameters were as follows: scan speed 60 nm/min, slit width 2 nm, wavelength step 0.21 nm. Second-order derivative spectra were calculated using the GBC 914 software package. The derivative spectra were smoothed with $\Delta\lambda$=5.0 nm.

Procedure for the calibration curve preparation

The calibration plot for determination of paracetamol was prepared by the following procedure: appropriate aliquots of paracetamol stock solution were diluted to 10 ml with double-distilled water to form standard solutions in the concentration range 5.0–30.0 μg/ml.

The UV absorption spectra were recorded and second derivative spectra computed. The amplitude $^2D_{246}$ was used for calibration curve

construction. The measurements were carried out in triplicate.

The limit of detection was experimentally determined using 2.0 μg/ml paracetamol aqueous solution. The second derivative spectra was obtained and the limit of detection determined by measuring the corresponding value of $^2D_{246}$ in comparison with the noise signal (measured in the wavelength range 350–400 nm).

Procedure for determination of paracetamol in urine samples

The urine samples, collected from healthy volunteers, were diluted 1:100. The zero-order spectra of all investigated samples were recorded against water in the wavelength range 220–400 nm. The second-order derivative spectra, obtained by digital differentiation, were utilised for determination of paracetamol using amplitude $^2D_{\lambda zc}$, where λ_{zc} is the wavelength corresponding to zero-crossing point in the particular second-order derivative spectra of blank urine sample.

In vivo experiment

Four healthy volunteers, two men and two women (aged 30–32, weight 66–85 kg, height 172–185 cm) participated the study. Each subject was informed about the purpose, protocol and risk of the study and gave written consent to participate. Subjects did not take any other medications or alcohol for at least 2 weeks prior to and throughout the study. Each subject fasted overnight prior to the experiment, and food was withheld for 4 h after dosing. The paracetamol solution, equivalent to 325 mg drug, (Paracet® syrup, Zdravlje, Yugoslavia) was taken with 200 ml of tap water. Subjects were instructed to take water after each urine collection to ensure adequate diuresis. Urine samples were collected prior to drug administration (blank urine samples) and 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 h after dosing. The total volume of urine voided over each time interval was measured and aliquots of each sample were frozen in labeled containers until the day of analysis. In the series of 12 samples drug concentration was determined prior to and after storage at −20°C for 4 weeks to evaluate the stability of frozen samples. In order to obtain cumulative urinary excretion data, the drug concentration determined in each urine sample was multiplied by the volume of urine excreted during the collecting period.

Results and Discussion

In clinical and biological analyses the effect of light scattering in turbid systems, which leads to nonspecific background absorption that increases towards the shorter wavelengths' could have a significant effect on analyte determinations. In such cases measurements of total absorption at a single wavelength would be grossly in error. Derivative spectroscopy can be used for determination of drug substances in biological fluids since in the second derivative spectrum the broad background interference is selectively suppressed. Also, the application of zero-crossing method could be useful in biological analysis.

In the first stage of method development, the urine samples collected from healthy volunteers, on different occasions (fed or fasted), in different time intervals over several days were analysed. The second-order derivative spectra of all sufficiently diluted urine samples, irrespective of their shape and intensity, displayed the presence of a zero-crossing point within the very narrow wavelength interval (245–247 nm) defined as λ_{zc} (Figure 1).

The zero-order absorption spectra of paracetamol in water displays maximum absorbance at 243 nm, while in the second derivative spectra, a broad minimum peak at 244–248 nm was obtained under defined experimental conditions and selected set of instrumental parameters. The existence of λ_{zc} in the spectra of blank urine samples, which is within the broad minimum peak in the second-order derivative spectra of aqueous paracetamol solution, enables the application of zero-crossing technique for paracetamol determination in urine samples using amplitude $^2D_{\lambda zc}$.

The addition of paracetamol to the blank urine sample resulted on a spectrum that was the simple sum of their separate spectra. The zero-order spectra of paracetamol in water, paracetamol added to blank urine and a blank urine sample are given in Figure 2, while the

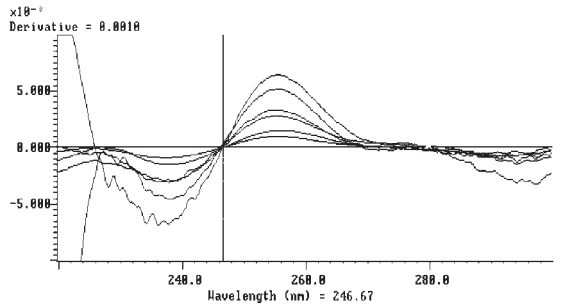

Figure 1. Second-order derivative spectra of blank urine samples collected from different volunteers on different occasions

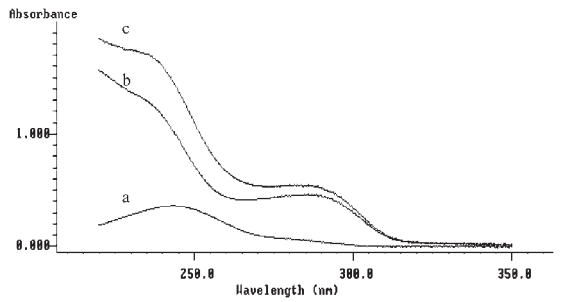

Figure 2. UV-absorption spectra of: (a) paracetamol in water (5 μg/ml); (b) blank urine and (c) urine spiked with paracetamol (5 μg/ml)

corresponding second-derivative spectra are presented in Figure 3.

Regarding the fact that paracetamol in urine is present in the form of conjugates, an attempt to obtain the fraction of total paracetamol, by applying enzyme hydrolysis, was made. An equivalent of 500 units of *β*-glucuronidase with sulphatase activity was added to 1 ml of urine samples collected after per oral application of Paracet® to the volunteers. The samples were incubated at pH 5.0 in a water bath at 37°C for 1 h (according to the method proposed by Vila-Jato *et al*. [13]). Comparison of second derivative spectra of samples with and without enzyme, prior and after incubation, did not show any significant differences that could be attributed to the increase in concentration of free paracetamol.

These findings indicated the possibility that not only the unchanged drug, but also the total paracetamol was detected using second order derivative UV-spectrophotometry. In order to examine and verify this hypothesis, an appropriate

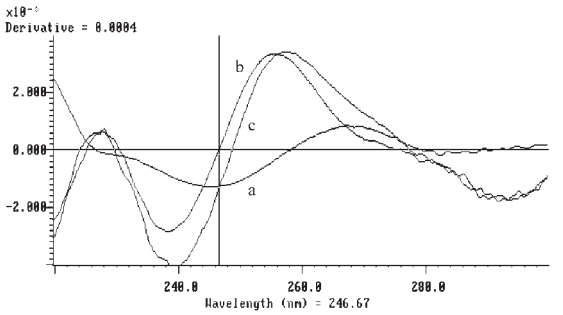

Figure 3. Second-order derivative spectra of: (a) paracetamol in water (5 μg/ml); (b) blank urine and (c) urine spiked with paracetamol (5 μg/ml)

aliquot of paracetamol stock solution was added *in vitro* to urine samples obtained after per oral application of paracetamol solution. Corresponding spectra were recorded and analysed. When comparing the second derivative spectra of samples with and without *in vitro* added paracetamol, certain shifts of satellite peaks could be observed, but the array of the spectra in which the zero-crossing of blank urine and working amplitude of paracetamol are located showed no significant differences. The only difference was an intensified signal corresponding to paracetamol in spiked samples. The obtained results indicated that the proposed method could be used for simple and direct determination of total paracetamol in urine.

In order to select the conditions for construction of the calibration plot, the influence of the medium should be considered. When observing second derivative spectra of paracetamol solutions in water, blank urine and a urine sample spiked with known amounts of paracetamol stock solution (Figure 3), it could be noticed that the amplitudes of both the paracetamol aqueous solution and the urine sample containing the same amount of paracetamol, at the zero-crossing wavelength corresponding to blank urine sample, were equal. The crossing point between the spectra of the spiked urine sample and the paracetamol aqueous solution of the same concentration (Figure 3, curves a and c), which is at the same wavelength where the zero-crossing point of the particular blank urine spectrum was found (λ_{zc}—indicated by the vertical line), confirms that spiked urine samples are not required for calibration curve construction. Therefore,

standard solutions of paracetamol in water were used for the calibration curve preparation and method validation (e.g. evaluation of detection limit and limit of determination). Regarding the fact that aqueous paracetamol solution displays a plateau over the wavelength range 244–248 nm, the measurements for calibration curve construction were performed on λ=246 nm, while measurements intended to determine paracetamol concentrations in investigated samples were performed on the wavelength corresponding to zero-crossing point of the particular subject. In this way the experimental error is minimised, since the slight differences in λ_{zc} of particular subjects were taken into account and accurate determination of paracetamol in investigated urine samples was accomplished.

The calibration curve was constructed by plotting the values of the amplitude $^2D_{246}$ (absolute amplitude value multiplied by 10^3, in order to simplify the regression equation) *vs* the concentration of paracetamol standard solutions. The obtained regression equation was:

$$^2D_{246} = 0.2512c + 0.0003$$

where $^2D_{246}$ is the absolute value $\times 10^3$ amplitude $^2D_{246}$, and c is paracetamol concentration (μg/ml).

The correlation coefficient of the calibration curve was 0.9995. The repeatability of the proposed method was evaluated for the solution containing 3.5 μg/ml of paracetamol and the obtained value of relative standard deviation (RSD) was 1.62% (ten replicate measurements). The experimentally obtained value for the limit of detection was 3.47 μg/ml, defined as the concentration giving an amplitude signal that is three times higher than the noise signal (SNR). The obtained RSD value indicated the experimentally determined limit of quantification of 3.5 μg/ml. The value of SNR of 5, which should be used for biological samples, was not applied because the zero-crossing method was employed.

In vivo study

Total paracetamol in urine was determined spectrophotometrically according to the proposed method. In order to examine the stability of paracetamol in urine samples, a drug concentration was determined prior to and after 4 weeks storage at −20°C, in a series of 12 samples collected from one of the volunteers according to the predefined time intervals. The obtained data were statistically evaluated using paired Student's *t*-test (two population). The results were found not to be significantly different at $p = 0.05$ ($n = 12$; $v = 11$; $t = 0.27219$; $t_{tab} = 2.201$). The cumulative percent excreted in urine at the specified time intervals were calculated. Cumulative urinary excretion plots for each volunteer are given in Figure 4. An average value close to 95% (88.86, 95.45, 97.00, 97.72) of the applied dose of paracetamol was found to be excreted in urine after 12 h.

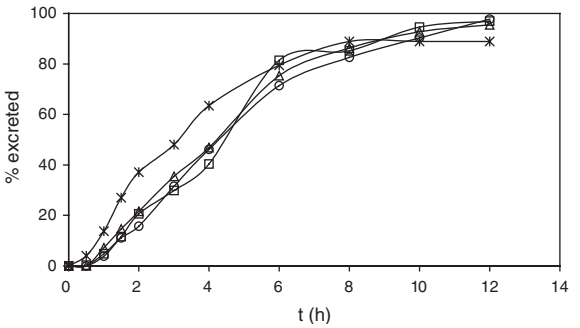

Figure 4. Cumulative percent of paracetamol excreted in urine after per oral administration of paracetamol syrup determined by the proposed method

The obtained results were in accordance with the data on cumulative urinary excretion after per oral administration of paracetamol solution reported by other authors [8, 11, 14] regarding both the cumulative amount excreted, as well as the time profile of the elimination; this provides further support for the hypothesis that the method described allows one to determine the total amount of paracetamol excreted (free plus conjugated).

The proposed method enables determination of total paracetamol in urine directly and simply by reading the $^2D_{\lambda zc}$ of the diluted samples obtained through the appropriate *in vivo* study under controlled conditions involving forced diuresis in the absence of all other medications, including the alcohol and caffeine. The advantage of derivative spectral measurements in clinical analysis includes the simplification of the analytical procedure as it allows the elimination of sample pretreatment, extraction

and derivatisation procedures. The reported procedure represents a non-invasive and cost effective method suitable for monitoring of the concentration of paracetamol in urine in bioavailability testing, for the evaluation of *in vitro–in vivo* correlation and screening of different formulation variables during drug product development.

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