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# Simultaneous determination of melatonin and pyridoxine in tablet formulations by Differential Pulse Voltammetry

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A method has been developed for the simultaneous determination of melatonin (MT) and pyridoxine hydrochloride (PY) in pharmaceutical dosage forms by differential pulse voltammetry, based on the oxidation of both drugs at a glassy carbon electrode. Cyclic and linear scan voltammetry were used to examine the influence of pH, nature of the buffer, scan rate and concentration. The results in 0.5 M $H_2SO_4$ with 20% methanol allowed a method to be developed for the determination of MT and PY simultaneously and in the presence of each other in the ranges $2 \times 10^{-5}$–$8 \times 10^{-5}$ and $2 \times 10^{-5}$–$4 \times 10^{-4}$ M, with the detection limits of $5.86 \times 10^{-6}$ and $2.45 \times 10^{-6}$ M, respectively. The proposed method was succesfully applied to the commercial tablets containing this drug combination without any interference by the excipients.

## 1. Introduction

Melatonin (MT) is well known as an indolaminergic hormone [1–3]. Pyridoxine hydrochloride [PY], one form of vitamin $B_6$, is an essential vitamin for humans [4]. The combination of MT with PY is used for regulating the body's natural sleep/wake cycle.

HPLC methods with UV [5], fluorescence [6–9], electrochemical [10–12] and MS [13] detection have been primarily used for the analysis of MT in biological samples such as blood, urine, pineal glands etc. Radioimmunoassay [13–15], GC [14, 16] and capillary electrophoresis (CE) [17] were also reported for the determination of the drug in several tissues and body fluids. Methods for the assay of MT in pharmaceutically dosage forms are usually based on GC-MS [16, 18], densitometric TLC [19], and fluorimetric [20, 21] determinations. To our knowledge, there is no official method for the drug in any pharmacopoeia.

A number of reports about the analytical methods of PY in either single or multicomponent mixtures describe HPLC [22–27] and CE [28–32] methods. In addition, potentiometry using bromide-selective electrode [33] and vitamin $B_6$ membrane electrode [34], spectrophotometry [35–38], TLC-spectrophotometry [39], and flow injection [40] were reported for the determination of the PY. The drug is included in USP 24 [41]. PY injection and tablets were determined with the official method, which involves a spectrophotometric procedure.

Voltammetry has also been applied for the determination of MT [42–45] and PY [46, 47], which are well known electroactive compounds, in pharmaceutical preparations and biological fluids.

In recent years, only few methods are reported for the simultaneous determination of these compounds in pharmaceutical dosage forms based on derivative spectrophotometry and spectrofluorimetry [48], derivative spectrophotometry [49] and capillary electrophoresis with electrochemical detection [50]. So far, we have not found any reports on the simultaneous determination of MT and PY in pharmaceutical preparations by voltammetry.

In this study, a systematic work, for the simultaneous determination of MT and PY in tablet dosage by differential pulse voltammetry at a glassy carbon electrode, was carried out, after having made a more detailed study of the electrochemistry of these two drugs over previously reported works. In this sense, several parameters, such as solution pH, nature of the buffer, scan rate, concentration of the drugs, were investigated.

## 2. Investigations, results and discussion

The voltammetric oxidation of MT and PY was investigated in the presence of different supporting electrolytes including $H_2SO_4$, phosphate and Britton-Robinson buffers of different pH values (1.5–11.5).

MT was irreversibly oxidised on glassy carbon electrode yielding two or three oxidation steps depending on pH (Fig. 1). Continued cycling of MT revealed that the appearence of a redox couple, producing a reduction wave and a re-oxidation wave, in lower positive potential region. By scanning with a new electrode to a potential lower than the oxidation potential of MT, the absence of reduction waves on the reverse scan, and subsequent re-oxidation waves on the forward scan, indicates that the redox couple is due to a product formed at higher potentials. If succesive cyclic scans are made, it can be observed that the main oxidation process at more positive potentials decreases as the number of scans increased, while the peaks of the redox couple augmented, since this couple arises from the product of the chemical follow-up reaction of MT oxidation, as defined by Kissinger and Heinemann [51]. It was also seen that the re-oxidation peak at low positive potential was presented on the initial scan when the electrode pretreatment was not sufficient, indicating the strong adsorption of the chemical product onto the electrode surface. In order to ensure that there was no contamination from the previous experiment, a background scan was always taken. A mechanism for the complex nature of the oxidation process of MT at carbon electrodes (especially carbon paste electrode) has already been proposed by Radi and Bekhiet [42], who carried out the cyclic voltammetry of MT in aqueous and non-aqueous media. The first oxidation peak became sharper with an increase in the concentration of MT, and was easily measurable. Hence all subsequent work was based on the measurement of the magnitude of this step.

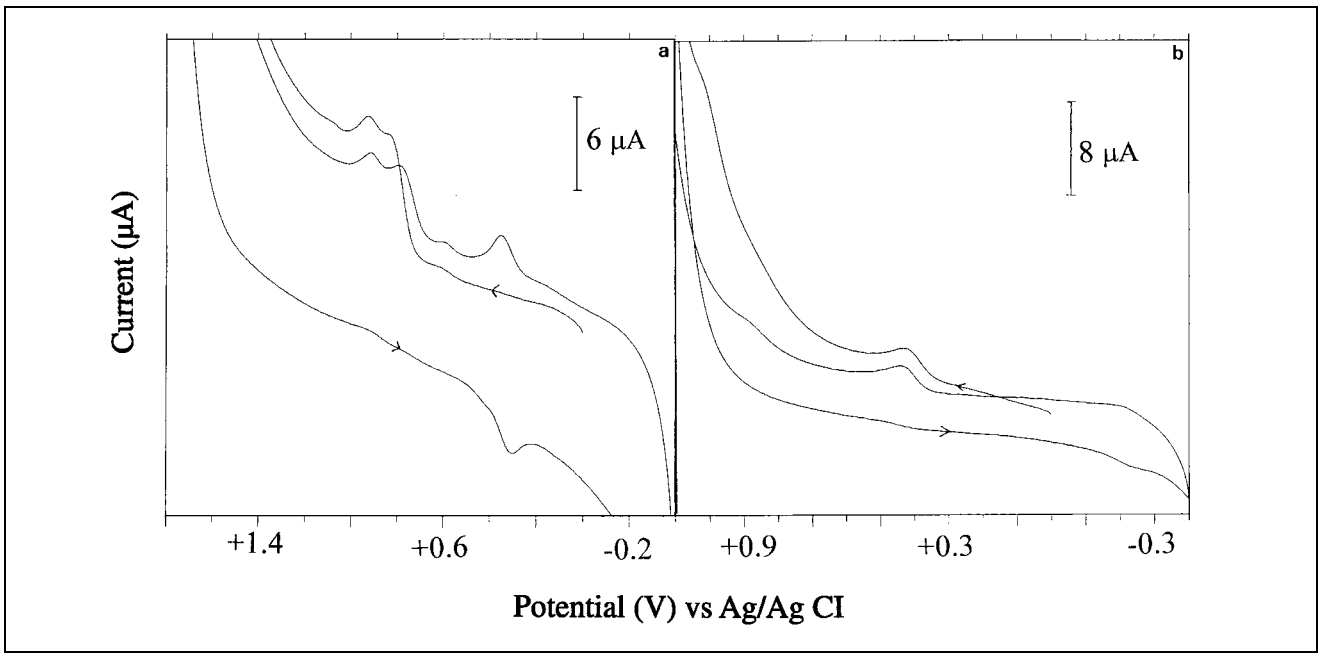


Fig. 1: Cyclic voltammograms of $2 \times 10^{-4}$ M melatonin in Britton Robinson buffer (20% methanol) at different pHs. Scan rate, 100 mV $s^{-1}$; (a) pH 2.15, (b) pH 11.02

PY was oxidized on glassy carbon electrode, producing only one anodic peak in acidic media (Fig. 2). At pH values greater than 6, this oxidation peak splitted into two steps, their shapes becoming broader.

The effect of the solution pH and the nature of the supporting electrolyte on the oxidation of both drugs using linear scan voltammetry was studied (Figs. 3, 4). The increase in pH produced a shift in the voltammograms of MT to less positive potential with a decrease in peak current, implying the involvement of protons in current-limiting electrode process. On plotting the peak potentials of MT against the pH value, a linear region was observed with a negative slope of 58.8 mV per pH unit (r: 0.995), corresponding to a Nernstian behavior involving a process with an identical number of protons and electrons, except

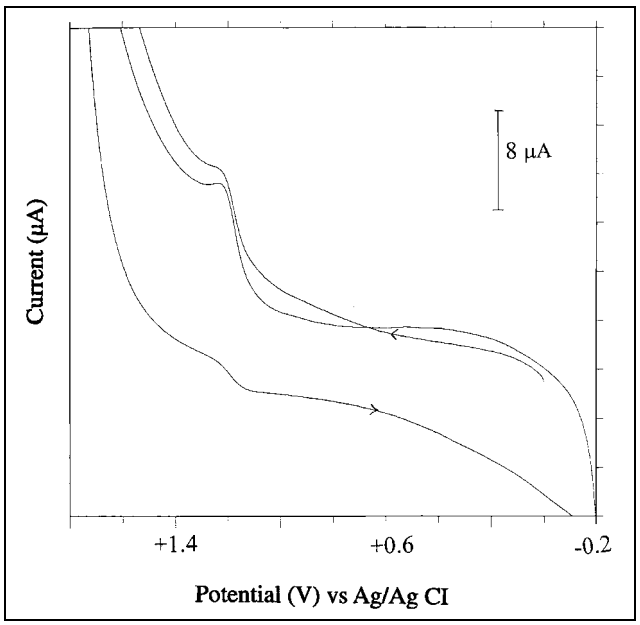


Fig. 2: Cyclic voltammogram of $2 \times 10^{-4}$ M pyridoxine in Britton Robinson buffer (20% methanol) at pH 2.15. Scan rate, 100 mV $s^{-1}$

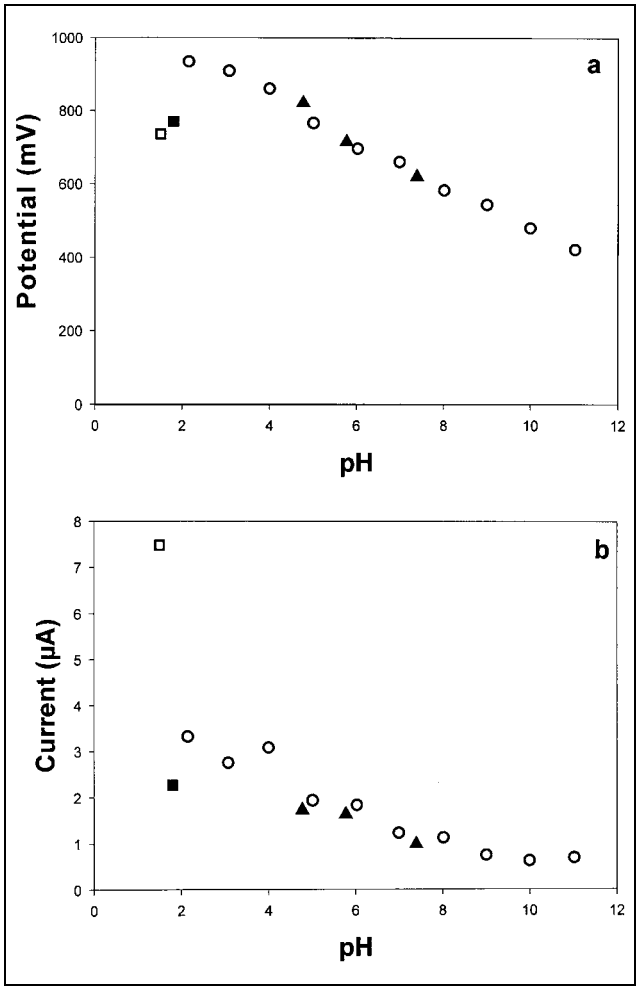


Fig. 3: Dependence of peak potential (a) and peak current (b) on pH. melatonin, $2 \times 10^{-4}$ M; Scan rate, 100 mV $s^{-1}$; (■) 0.1 M $H_2SO_4$, (□) 0.5 M $H_2SO_4$, (▲) phosphate buffer, (O) Britton-Robinson buffer

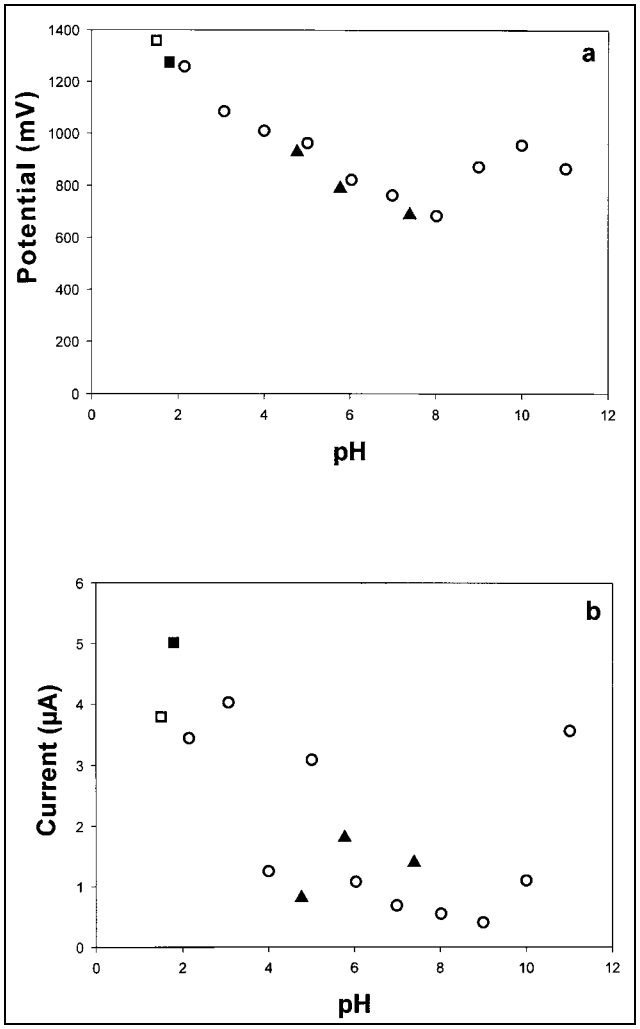


Fig. 4: Dependence of peak potential (a) and peak current (b) on pH. pyridoxine, $2 \times 10^{-4}$ M; Scan rate, 100 mV $s^{-1}$; (■) 0.1 M $H_2SO_4$, (□) 0.5 M $H_2SO_4$, (▲) phosphate buffer, (O) Britton-Robinson buffer

that in sulphuric acid which provides easier oxidation than buffer solutions. This shift is likely to be due to the influence of the buffer solutions on MT, suggesting that there is an interaction between the molecule and one of the buffer constituents. Further studies of the redox couple in the whole pH range investigated, show that the peaks are more defined and show a current increase with acidity. Additionally, the separation between cathodic and anodic peaks increased by raising pH ($\Delta E_p = 40$ and 53 mV at pH 2.15 and 4.0). In the case of PY, the peak potential decreased linearly with increasing pH, with a slope of 100.2 mV $pH^{-1}$ (r: 0.998), except for a inflection between pH 8 and 10.

The effect of the potential scan rate (ν) on the peak current ($i_p$) or the peak potential was also studied in the range 10–2000 mV $s^{-1}$. By plotting log $i_p$ against log ν, linear relation was observed with a slope of 0.586 (r: 0.996) between 10 and 1000 $mVs^{-1}$ for MT and 0.495 (r: 0.997) between 10 and 100 $mVs^{-1}$ for PY. These slopes are in close agreement with a slope of 0.5 that is to be expected for ideal reaction of the solution species [52]. Furthermore, the linear relationship existing between $i_p$ and the $\sqrt{\nu}$ for MT (r: 0.997) and PY (r: 0.997) showed the diffusion control process.

In order to develop a voltammetric method for determining the drugs, we selected the differential pulse mode,

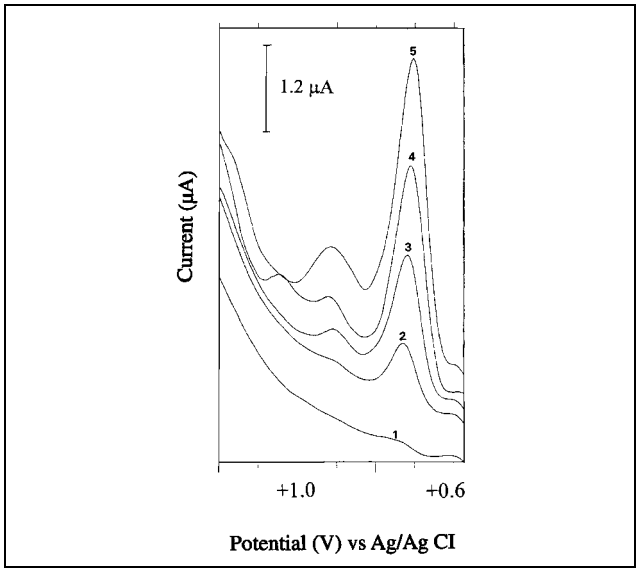


Fig. 5: Differential pulse voltammograms of different concentrations of melatonin in 0.5 M $H_2SO_4$ (20% methanol). Melatonin concentration, (1) 0 M, (2) $2 \times 10^{-5}$ M, (3) $4 \times 10^{-5}$ M, (4) $6 \times 10^{-5}$ M, (5) $8 \times 10^{-5}$ M

since the peaks were sharper and better defined at drug concentrations lower than those obtained by linear sweep voltammetry, with a lower background current, resulting in improved resolution. Knowing that 0.5 M sulphuric acid not only resulted in MT oxidation peak of greatest intensity but also gave the best peak shape for both drugs, experiments were carried out in this electrolyte. A pulse amplitude variation indicated 50 mV to result in the most intense peaks. A pulse width of 0.05 s gave rise to the sharpest and symmetrical peak shape. The optimum scan rate was 20 $mVs^{-1}$. It was found that the parameters had little effect on the peak potential.

The reproducibility of peak potentials and peak currents were tested by repeating four experiments on $8 \times 10^{-5}$ M MT and $6 \times 10^{-5}$ M PY. The relative standard deviations (RSD) were calculated to be 0.86 and 1.67% for peak currents and 0.46 and 0.37% for peak potentials for MT and PY, respectively.

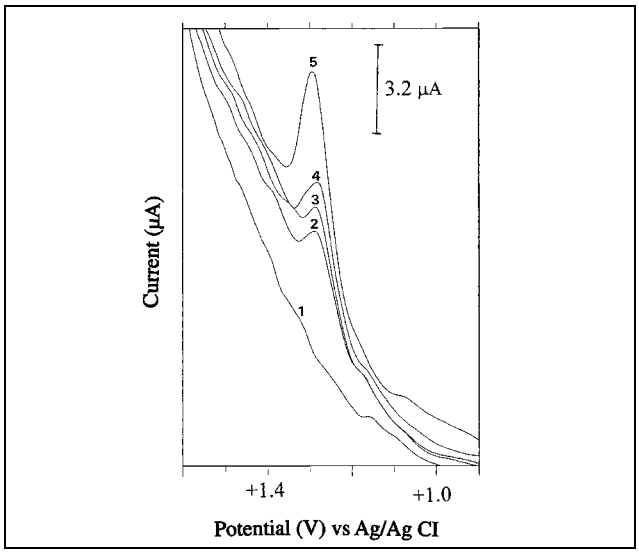


Fig. 6: Differential pulse voltammograms of different concentrations of pyridoxine in 0.5 M $H_2SO_4$ (20% methanol). Pyridoxine concentration, (1) 0 M, (2) $6 \times 10^{-5}$ M, (3) $8 \times 10^{-5}$ M, (4) $1 \times 10^{-4}$ M, (5) $2x10^{-4}$ M

**Table 1: Statistical analysis of melatonin (MT) and pyridoxine (PY) determinations in 0.5 M $H_2SO_4$ with 20% methanol using DPV**

| Sample | Measured potential (V) | Concentration range (M) | Slope ($\mu A\ M^{-1}$) | Intercept ($\mu A$) | Correl. coeff. | RSD of slope | RSD of intercept | Detection limit (M) | Determination limit (M) |
|---|---|---|---|---|---|---|---|---|---|
| MT | 0.72 | $2 \times 10^{-5}$–$8 \times 10^{-5}$ | $4.12 \times 10^4$ | 0.044 | 0.998 | 0.89 | 0.78 | $5.86 \times 10^{-6}$ | $1.95 \times 10^{-5}$ |
| PY | 1.29 | $2 \times 10^{-5}$–$4 \times 10^{-4}$ | $3.25 \times 10^4$ | −0.30 | 0.999 | 1.29 | 1.52 | $2.45 \times 10^{-6}$ | $8.15 \times 10^{-6}$ |

Typical differential pulse voltammograms obtained at various concentrations of MT and PY are shown in Figs. 5 and 6. The characteristics of the calibration graphs using the optimum conditions, are shown in Table 1.

The results show that MT and PY can be determined in the presence of each other because of the wide separation in their peak potentials. As it can be seen in Fig. 7, the current responses of MT and PY are separated with a potential difference of 570 mV.

In order to access the validity and applicability of the proposed method, recovery studies were performed by analysing synthetic mixtures of each drug in different composition ratios. According to these results obtained from synthetic mixtures, the mean recoveries and their RSDs were found to be 99.38 and 0.52% for MT and 98.75 and 0.97% for PY.

On the basis of these results, the proposed DPV was applied to direct and simultaneous determination of these drugs in commercial tablet form. The declared composition of the tablets were: MT (3 mg) and PY (10 mg) as principal components, and dicalcium phosphate, microcrystalline cellulose, glyceryl monostearate and magnesium monostearate as additional ingredients. As far as we know, a MT-PY mixture analysis is not yet official in any pharmacopoeia. For this reason, the simple derivative spectrophotometric method 49 was used for comparison. Table 2 compares the results of the analysis of MT and PY between proposed and literature method. The amounts of MT and PY found in tablet dosage forms are fairly close to the labeled amounts for both methods. The results obtained from the two methods were statistically compared with each other at the 95% confidence level with the aid of t- and F- tests. There is no significant difference between the two methods with respect to mean values and standard deviations since the calculated t- and F-values were less than the corresponding theoretical ones.

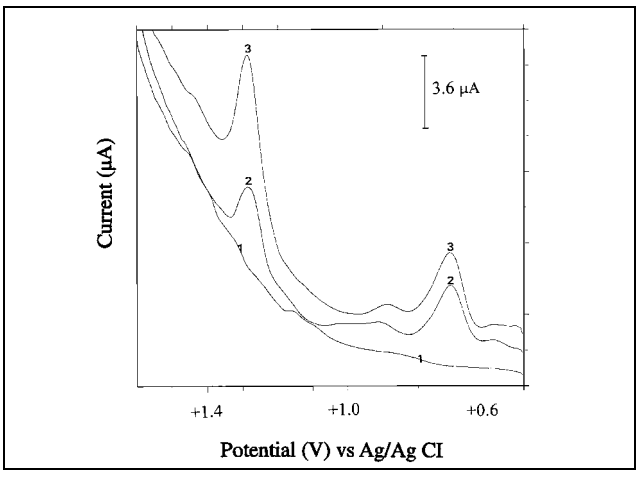


Fig. 7: Differential pulse voltammograms of melatonin-pyridoxine mixtures in 0.5 M $H_2SO_4$ (20% methanol). (1) Supporting electrolyte, (2) $6 \times 10^{-5}$ M melatonin + $1 \times 10^{-4}$ pyridoxine, (3) $8 \times 10^{-5}$ M melatonin + $2 \times 10^{-4}$ M pyridoxine

**Table 2: Comparison of the MT and PY determination results received by DPV and derivative spectroscopy**

| | DPV | | Derivative Spectrophotometry [49] | |
|---|---|---|---|---|
| | MT | PY | MT | PY |
| Labelled claim/mg | 3.00 | 10.00 | 3.00 | 10.00 |
| Amount found*/mg | 2.91 | 10.05 | 2.95 | 9.84 |
| RSD% | 1.79 | 1.55 | 3.53 | 2.44 |
| $t_{calc}$: | 0.69 | 1.62 | $t_{theor}$: 2.31 | |
| $F_{calc}$: | 0.21 | 0.42 | $F_{theor}$: 2.60 | |

*Each result is the mean of five experiments

In order to whether the excipients show any interference with the analysis, known amounts of the pure drugs were added to different pre-analysed formulations of MT and PY and the mixtures were analysed by the proposed method.

According to the results, the recoveries and their RSDs were found to be 99.26 and 0.58% for MT and 98.53 and 1.09% for PY, respectively.

On the other hand, the principal advantage of the proposed differential pulse voltammetric method over the other published procedures [48, 50] for MT-PY formulations is that it involves no sample preparation other than dissolving and transfering an aliquot to the supporting electrolyte and does not require separation procedures such as extraction and filtration, that are needed for spectrofluorimetric [48] or CE [50] procedures.

In conclusion, the voltammetric method developed in this study is easy to be carried out for the reliable analysis of combination of MT and PY either in a pure form or in tablets.

## 3. Experimental

### *3.1. Apparatus*

Electrochemical experiments were performed on a Bioanalytical Systems (West Lafayette, IN, USA) BAS 100 W electrochemical analyser. The three electrode system consisted of a glassy carbon working electrode (∅: 3 mm, BAS), an Ag/AgCl reference electrode (NaCl 3M, BAS) and a platinum wire auxillary electrode. The polishing of the glassy carbon electrode with aluminium oxide (∅: 0.01 μm) in the presence of doubly distilled $H_2O$ on a smooth polishing cloth, and rinsing with doubly distilled $H_2O$ in an ultrasonic bath, were carried out before each analysis.

For comparison study, derivative spectrophotometric measurements were carried out using a Shimadzu 1601 PC double beam UV-Vis spectrophotometer.

### *3.2. Chemicals, reagents and solutions*

MT and PY were purchased from Sigma (St.Louis, MO, USA) and used without further purification. All other chemicals used were of analytical grade quality (Merck and Sigma).

Stock solutions under voltammetric investigation were prepared daily by dissolution in MeOH. Standard solutions were prepared by dilution of the stock solution and contained 20% MeOH. $H_2SO_4$ (0.1, 0.5 M), phosphate buffer (0.2 M, pH 4.5–7.5) and Britton-Robinson buffer (0.04 M, pH 2.0–11.5) were used for the supporting electrolyte. All solutions were protected from light and were used within 24 h to avoid decomposition.

### *3.3. Analysis of tablets*

Ten tablets were weighed and powdered. Portion equivalent to stock solutions of a concentration about $10^{-3}$ M for each component was accurately

weighed, transferred into separate 100 ml volumetric flasks and dissolved in MeOH. The contents were allowed to settle after sonicating for 15 min. Appropriate solutions were prepared by taking suitable aliquots of the clear supernatant liquor and diluting them with supporting electrolyte in order to obtain a final solution of MeOH – 0.5 M $H_2SO_4$ (2 + 8 v/v). Each solution was transferred to a volumetric cell and recorded as in pure drugs.

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