# VOLTAMMETRIC BEHAVIOR OF 4-ACETAMIDOHIPPURIC ACID AND 4-ACETAMIDOBENZOIC ACID ON A DISPOSABLE CARBON ELECTRODE AND THEIR DETERMINATION IN HUMAN URINE

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The voltammetric behavior of 4-aminobenzoic acid (PABA) and its acetylated metabolite on glassy carbon, carbon fiber or carbon paste electrodes was investigated in an aqueous supporting electrolyte. 4-Aminohippuric acid (4-AHA), 4-acetamidobenzoic acid (4-AMB) and 4-acetamidohippuric acid (4-Ac-AHA) can be separated on a capillary carbon paste electrode in 0.1 M lithium perchlorate. The oxidation potentials of PABA, 4-AHA, 4-AMB and 4-Ac-AHA were 0.70, 0.88, 1.06 and 1.10 V on capillary CPE, respectively. The electrooxidation process is used for simultaneous quantitative determination of acetylated metabolites in urine.

**Keywords**: 4-Acetamidohippuric acid; 4-Acetamidobenzoic acid; Carbon electrodes; Cyclic voltammetry; Differential pulse voltammetry; Electrochemistry.

4-Aminobenzoic acid (PABA) and its acetylated metabolite 4-acetamidobenzoic acid (4-AMB) inhibit agonist-induced aggregation and arachidonic acid-induced [$Ca^{2+}$] transients in human platelets[1–4]. The literature presents several methods for the determination of 4-AMB [5–13]. Fluorescein-labelled[5] and radiolabelled[6] 4-AMB, and UV detection are combined with high-performance liquid chromatographic assay for the determination of PABA and its conjugates in human urine[7–9]. Determination of uric acid and 4-aminohippuric acid (4-AHA) in human saliva and urine using capillary electrophoresis with electrochemical detection[10,11]. High-performance liquid chromatography has been proposed for simultaneous determination of PABA and its metabolites in biological fluids but they need a relatively long chromatographic retention time of 25 min. Nevertheless, there were only two electrochemical methods applied to PABA determination which included electrochemical homopolymerization of PABA on Pt electrodes[12] and on covalently modified glassy carbon paste[13]. To our knowledge, the



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voltammetric behavior of 4-AMB and 4-acetamidohippuric acid (4-Ac-AHA) has not yet been investigated. Therefore, the electrochemical behavior of acetylated metabolites using a bare glassy carbon electrode (GCE), carbon fiber and carbon paste electrode has been investigated in various supporting electrolytes by cyclic (CV) and differential pulse voltammetry (DPV). The optimum experimental conditions for the simultaneous determination of acetylated metabolites containing human urine sample are described.

## EXPERIMENTAL

### Apparatus and Materials

Cyclic and differential pulse voltammetric experiments were performed using an EG&G Model 394 (Princeton Applied Research, Princeton, NJ, USA) connected to an EG&G325 Faraday cage with Smart stir and KO269 A Faraday cage. A three-electrode system was employed, consisting of a working electrode (glassy carbon, carbon fiber and carbon paste electrode), a platinum counter and an Ag|AgCl reference electrode. A glassy carbon electrode (GCE) and a rotating disk (o.d. 3 mm, Metrohm) were used. 4-Aminobenzoic acid (PABA), 4-aminohippuric acid (4-AHA) and 4-acetamidobenzoic acid (4-AMB) were obtained from Acros Organics (New Jersey, USA), 4-acetamidohippuric acid (4-Ac-AHA) was synthesized from 4-AHA [14]. All other reagents were of analytical grade. Structures of these compouds are shown in Fig. 1

### Procedure

*Fabricating a disposable electrode*. A bundle of polyacrylonitrile carbon fibers (PAN type) with 0.16–0.32 μm diameter (obtained from Formosa Synthetic Fiber Research Institute)

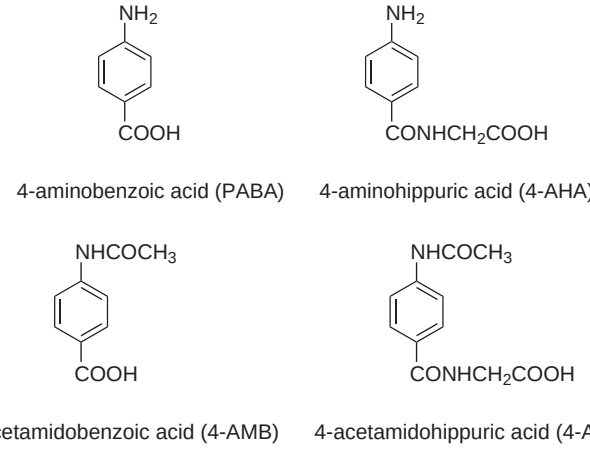


FIG. 1
Chemical structures

was inserted into one end of Teflon tube and sealed with acrylic resin (obtained from Struers). A small copper wire was inserted in the other end of the Teflon tube to enable electrical connection to the fiber. A typical carbon paste preparation procedure was as follows: 1.2 g of graphite powder (Merck) and 0.8 g of liquid paraffin (Merck) were mixed in a mortar. The body of the carbon paste working electrode was fabricated from a capillary glassy rod (o.d. 0.8 mm; Kimax, USA) with a 10 mm hole bored into one side for the carbon paste filling. The carbon paste was placed into the body of the electrode with a PTFE spatula, and smoothed off.

*Determination of PABA and acetylated metabolites by voltammetry*. The Britton–Robinson buffer solutions (pH 2.56, 4.27, 5.09, 6.15, 7.11 and 8.74) were prepared by mixing 0.5 M phosphoric acid, 0.5 M boric acid, 0.5 M acetic acid and 0.2 M sodium hydroxide solutions; pH was checked by a pH meter. Voltammograms were taken for PABA and acetylated metabolites in a phosphate buffer (pH 2.25 and 6.08), 0.1 M acetate buffer (pH 4.89), Britton–Robinson buffer and methanol or water containing various supporting electrolytes such as sodium perchlorate, lithium perchlorate and tetraethylammonium tetrafluoroborate.

In order to obtain calibration plots for the 4-AMB and 4-Ac-AHA, 10 ml of supporting electrolyte was pipetted into a voltammetric cell. Aliquots of 1000 ppm (mg $l^{-1}$) 4-AMB and 4-Ac-AHA solutions were added. After each addition voltammograms were obtained. For the pre-concentration step, the working electrode was placed in 10 ml of stirred test solution for 120 s. Quantative analyses were performed in the differentical pulse mode. The potential was set at 0.4 to +1.4 V vs Ag|AgCl. The pulse height was 50 mV and the scan rate 10 mV $s^{-1}$ with a drop time of 1.0 s. For solution analysis, 0.1 ml of the solution was pipetted into a 10-ml calibrated flask and diluted to the volume with phosphate buffer. In order to obtain reproducible results, a standard pre-treatment procedure was applied before each voltammogram. The previous sample solution was rinsed from the electrode and the surface then renewed by removing the outer 2 mm and refilling with fresh carbon paste. The solution was analyzed by DPV using the same conditions as for the calibration plot.

## RESULT AND DISCUSSIONS

### *Voltammetric Behavior of PABA and Its Acetylated Metabolites*

Aromatic carboxylic acids and their derivatives can be reduced at very negative potentials[15]. However, aromatic amines are readily oxidized on the anode in aqueous and organic solvents. Usually, potentials of 0.5–1.5 V vs SCE are adequate for the generation of primary cation radicals of amino compounds[16]. Therefore, the oxidations of PABA, 4-AHA, 4-AMB and 4-Ac-AHA were studied on carbon fiber electrode (CFE), carbon paste electrode (CPE) and glassy carbon electrode (GCE) by differential pulse voltammetry (DPV), respectively. As can be seen in Table I, the detection limits of PABA, 4-AHA, 4-AMB and 4-Ac-AHA were 1.29, 5.51, 2.66 and 4.61 mg $l^{-1}$ on CPE, respectively, which had lower detection limits than the other electrodes. The CPE using graphite powder mixed with an organic binder (e.g. paraffin oil), offers the advantages of low background current

and noise levels, low cost, and an easily renewable surface. The extractive adsorption accumulation of organic compounds on carbon paste electrodes, which use a pre-concentration step prior to voltammetric measurement; hence, the CPE has the advantage of being more sensitive than the other techniques. Therefore, the CPE was chosen for use in the determination of PABA and its acetylated metabolites. The oxidation potentials of PABA, 4-AHA, 4-AMB and 4-Ac-AHA were 0.70, 0.88, 1.08 and 1.18 V on capillary CPE, respectively. Amides are a special type of substituted amines. In general, they are oxidized on the anode, but at considerably more positive potentials than the amines. The lone pair of the nitrogen atom is delocalized on the carbonyl group, which makes these electrons less available for electron release to the anode. It is likely that amines are more prone to be adsorbed on the anode than the amides[17,18]. Therefore, the potential of 4-AMB (1.08 V) is more positive than PABA (0.70 V), much the same as 4-Ac-AHA (1.18 V) is more positive than the 4-AHA (0.88 V). The steric effect of $CONHCH_2COOH$ on the benzene ring is larger than of COOH. As would be expected 4-AHA (0.88 V) requires more positive potentials than PABA (0.70 V). Similarly 4-Ac-AHA (1.18 V) requires more positive potentials than the 4-AMB (1.08 V).

TABLE I
Detection limit of PABA and its metabolites on glassy carbon, carbon fiber and carbon paste electrodes at differential pulse voltammetry

| Electrode | PABA, mg $l^{-1}$ [b] | 4-AHA, mg $l^{-1}$ [b] | 4-AMB, mg $l^{-1}$ [b] | 4-Ac-AHA, mg $l^{-1}$ [b] |
|---|---|---|---|---|
| GCE | 4.93 | 8.68 | –[a] | –[a] |
| CFE | 3.05 | 5.97 | 3.25 | 6.21 |
| CPE | 1.29 | 5.51 | 2.66 | 4.61 |

[a] Not determined. [b] Concentration: ppm (mg $l^{-1}$).

### *Effects of pH and Supporting Electrolyte*

Several electrolytes (0.1 M phosphate buffer, pH 2.25 and 6.80; Britton–Robinson buffer, pH 2.56–8.74; tetraethylammonium tetrafluoroborate, pH 2.55; 0.1 M acetate buffer, pH 4.89 and 0.1 M lithium perchlorate, pH 6.94 were tested to find the optimum pH for separation of the pathological metabolites of PABA (Fig. 2). The differential pulse voltammetric peak potential and peak current are affected by pH and supporting electrolytes (Table II). It can be seen that lithium perchlorate is the most suitable since

TABLE II

Effect of pH and supporting electrolytes at the differential pulse voltammetric peak potential and peak current of PABA, 4-AHA, 4-AMB and 4-Ac-AHA on capillary carbon paste electrodes (i.d. 0.8 mm) at the standard concentration of 4 mg $l^{-1}$

| Supporting electrolyte | pH | PABA | | 4-AHA | | 4-AMB | | 4-Ac-AHA | |
|---|---|---|---|---|---|---|---|---|---|
| | | potential V | current nA | potential V | current nA | potential V | current nA | potential V | current nA |
| Phosphate buffer | 2.25 | 0.85 | 91.3 | 0.87 | 129 | 1.02 | 160 | –[a] | –[a] |
| Tetraethylammonium tetrafluoroborate solution | 2.55 | 0.78 | 185 | 0.80 | 59.7 | 1.21 | 344 | 1.23 | 451 |
| Acetate buffer | 4.89 | 0.68 | 60.1 | 0.77 | 141 | 1.05 | 339 | 1.23 | 98.8 |
| Phosphate buffer | 6.80 | 0.65 | 133 | 0.70 | 72.4 | 1.02 | 131 | 1.21 | 221 |
| Lithium perchlorate solution | 6.94 | 0.70 | 125 | 0.88 | 30.0 | 1.08 | 635 | 1.18 | 779 |
| BR buffer | 5.46 | 0.69 | 741 | –[a] | –[a] | 1.04 | 478 | 1.13 | 268 |

[a] Not determined.

the peak currents of 4-AMB and 4-Ac-AHA are higher than the others. As can be seen in Fig. 2, it improves the separation of the three peaks.

Cyclic voltammograms were recorded at different scan rates as shown in Fig. 3. The anodic current ($i_{p,a}$) depends on the square root of scan rate ($v^{1/2}$) and cathodic current ($i_{p,c}$) is zero; thus ($i_{p,a}$)/($i_{p,c}$) is zero for all scan rates. The fact that no peaks were observed in the cathodic direction suggests that the process is irreversible. The current function values ($I_p = i_p/A\,C_A\,v^{1/2}$) in Britton–Robinson buffer (where $i_p$ is peak current, $A$ is the area of electrode, $C_A$ is the concentration and $v$ is the scan rate) are constant at low concentrations (4 ppm) and low scan rates (10 mV $s^{-1}$) of 4-AMB. The $i_p$ is proportional to $v^{1/2}$ (2.0–50 mV $s^{-1}$) and $C_A$ (4–64 ppm) (Fig. 4). A good linear relationship was observed between the peak height (current) and the square root of scan rate. The regression equation is $y = 1.69X - 0.11$ (correlation coefficient $R = 0.9988$) for the square root of scan rate. This type of electrochemical behavior should correspond either to diffusion controlled irreversible charge transfer or a reversible charge transfer followed by an irreversible fast chemical reaction[19]. The relationship between the peak potential and logarithm of the scan rate can be used for approximate estimation of the number of electrons involved in the catalytic oxidation.

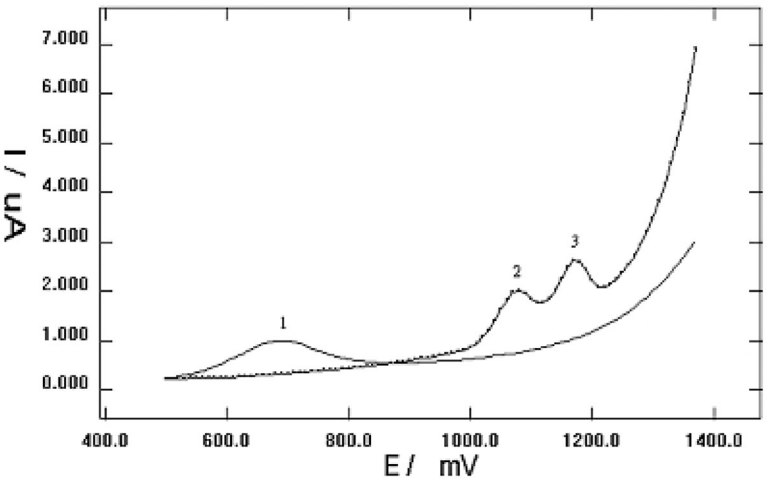


FIG. 2

Separation of PABA metabolites by DPV method: 1 4-AHA (0.74 V), 2 4-AMB (1.08 V), 3 4-Ac-AHA (1.18 V) in a capillary tube with carbon paste, pulse height 0.05 V. 0.1 M lithium perchlorate containing 32, 4 and 4 ppm (mg $l^{-1}$) of 4-AHA , 4-AMB and 4-Ac-AHA, respectively

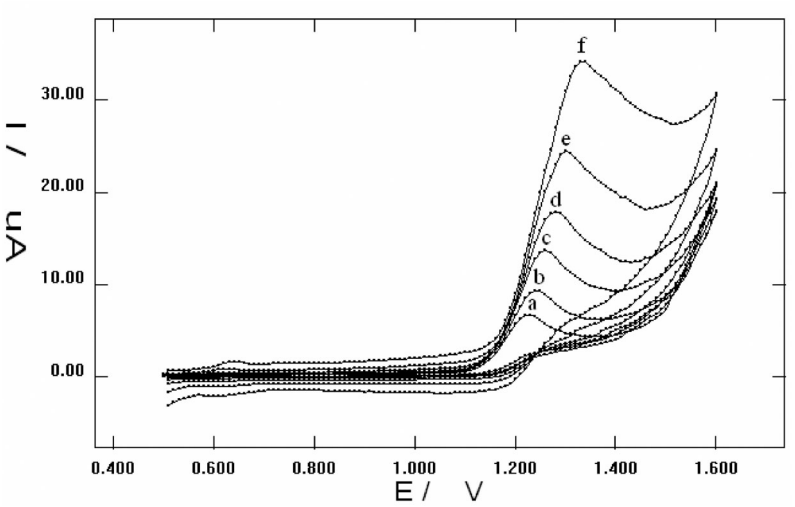


FIG. 3
Cyclic voltammograms of 4-AMB ($1 \times 10^{-2}$ mol $l^{-1}$) on CPE in Britton–Robinson buffer (pH 5.09) at different scan rates (in mV $s^{-1}$): a 12.5, b 25, c 50, d 100, e 200, f 400

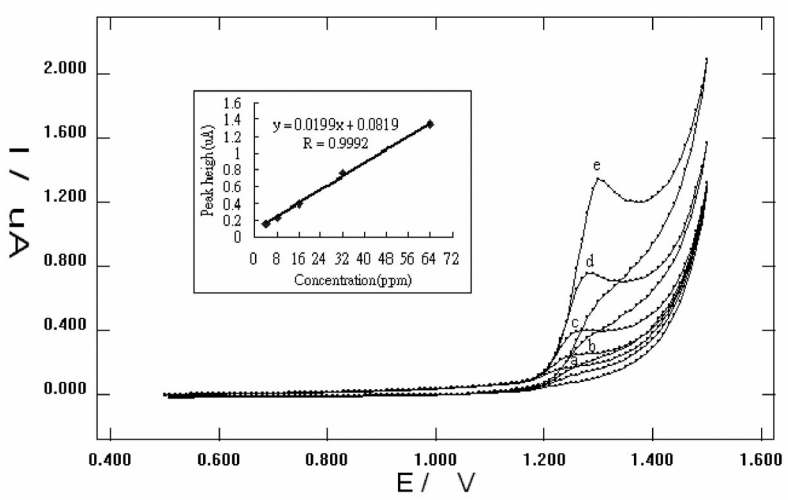


FIG. 4
Cyclic voltammograms of the oxidation of 4-AMB on a capillary CPE in 0.1 M $LiClO_4$ solution at scan rate 10 mV $s^{-1}$. Concentration (in ppm): a 4 (1.23 V, 0.156 μA), b 8 (1.24 V, 0.232 μA), c 16 (1.26 V, 0.3940 μA), d 32 (1.28 V, 0.7531 μA), e 64 (1.3 V, 1.34 μA)

*Application to Analysis of Human Urine*

Methods of quantification of 4-AMB and 4-Ac-AHA based on the above voltammetric studies were described in this section. Their results show that adsorptive preconcentration of 4-AMB and 4-Ac-AHA on the CPE allows a significant enhancement of the electrocatalytical effects. Two well-defined peaks at 1.08 and 1.18 V can be recorded after 60 s of preconcentration on the CPE (Fig. 5). Samples of human urine (1 ml) were centrifuged at 3000 *g* for 30 min. The supernatant urine was transferred to another centrifugal tube containing 2 ml of ethanol and centrifuged for 30 min to sediment the aggregates. The deproteinized samples were then extracted three times using 5–15 ml of chloroform. The organic phase was collected and evaporated under nitrogen at a temperature less than 37 °C. The dried extract was reconstituted with 10 ml of methanol and aliquot of 0.2 ml sample solution was added to a polarography vessel. The qualitative and quantitative results were obtained using a standard addition method in a polarographic

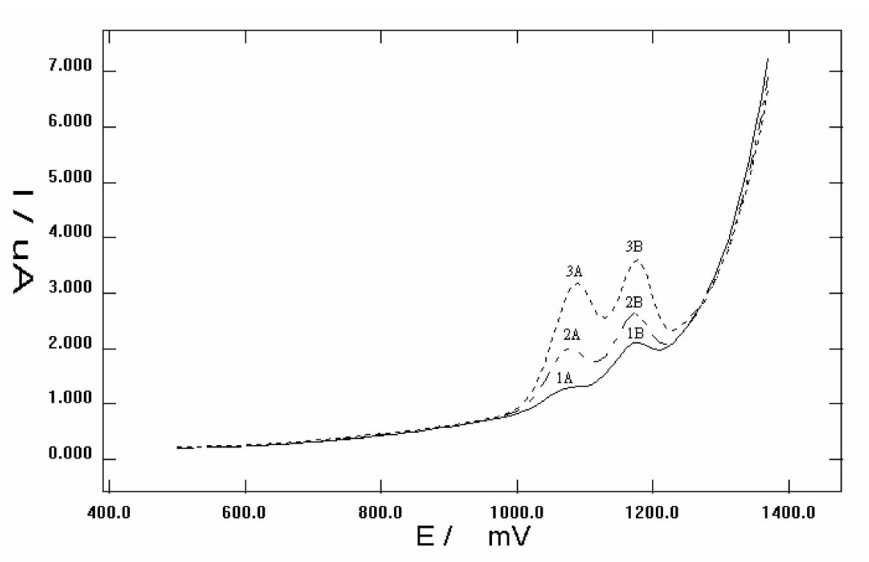


FIG. 5

Differential pulse voltammograms of 4-AMB (A) and 4-Ac-AHA (B) in 0.1 M lithium perchlorate in a capillary tube with carbon paste. Current peak values: 1A 1.31 μA (1.08 V, 1 ppm), 1B 2.11 μA (1.18 V, 1 ppm); 2A 2.01 μA (1.08 V, 2 ppm), 2B 2.62 μA (1.18 V, 2 ppm); 3A 3.17 μA (1.09 V, 4 ppm), 3B 3.60 μA (1.18 V, 4 ppm). Scan rate 10 mV $s^{-1}$, pulse height 0.05 V. The pre-concentration step, the working electrode placed in solution for 120 s

cell. The proposed DPV method was also applied to the determination of PABA, its acetylated metabolite in human urine. The major metabolite determined using this approach was 4-AMB, and the minor metabolites were 4-Ac-AHA and 4-AHA. Very small amounts or without of 4-Ac-AHA and 4-AHA were determined in urine which the same as previous study[20]. A representative DPV voltammogram of a urinary metabolite of 4-AMB after 32-h application of 5% PABA cream to human skin by standard addition method is shown in Fig. 6. The peak current increases linearly with the analytical concentration of 4-AMB over the range 1–32 mg $l^{-1}$. The correlation coefficient *R* of the calibration line over this range is 0.9936.

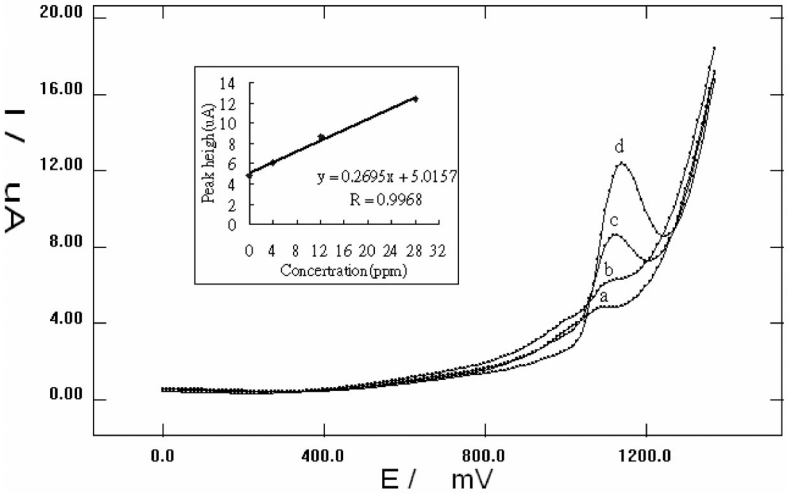


FIG. 6

Differential pulse voltammograms of urinary metabolites of 4-AMB after 32-h application of 5% PABA cream to human skin on a CPE. Peaks: a 1.080 V, 4.790 μA (0 ppm 4-AMB added); b 1.100 V, 6.110 μA (4 ppm 4-AMB added); c 1.120 V, 8.620 μA (12 ppm 4-AMB added); d 1.140 V, 12.40 μA (28 ppm 4-AMB added). Scan rate 10 mV $s^{-1}$, pulse height 0.05 V. The pre-concentration step, the working electrode placed in solution for 120 s

## *Conclusion*

The DPV procedures described here are applied directly to the analysis of human urine without the need for prior separation of PABA and its acetylated metabolites. A 1200 mV scan is run once, which takes 2 min at 10 mV $s^{-1}$. This procedure not only provides a better sensitivity and accuracy but also is less time-consuming. In comparison with non-disposable

counterparts, disposable electrodes, CPE offer improved simplicity and low cost.

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