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Characterization of the composition of bovine urine and its effect on the electrochemical analysis of the model mediator, *p*-aminophenol

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Abstract

The reliable identification of compounds such as illegal growth promoters in cattle is generally based on expensive gas chromatography–mass spectrophotometric analysis in urine, a method that does not allow on a large-scale screening. The use of simple, semi-quantitative electrochemical biosensors may provide a means of screening for the presence of compounds such as illegal growth promoters. Before such sensors can be utilised, it is necessary to understand which factors influence the response of an electrochemical sensor in bovine urine. The concentration range of protein (0.01–0.04%), uric acid (0.5–0.65 mM), xanthine (0.02–0.12 mM) and ascorbic acid (0.1–0.95 mM) in 26 individual urine samples were determined. Using *p*-aminophenol (*p*-AP) as a model system, the electrochemical response increased by 5% in the presence of 6.0 mM uric acid, by 10% on the addition of 0.2 mM xanthine and by 22% in the presence of 1.0 mM ascorbic acid. Exposing urine to air and light for 75 min eliminated interference from ascorbic acid. Addition of Cu^{2+} (10 μM) reduced the time required to 34 min. Binding of species such as growth promoters to proteins may be disrupted by the addition of 8-anilino-1-naphthalene sulphonic acid (ANS) to the urine samples. Addition of 10 μM ANS did not affect the limit of detection of *p*-AP. The pH of fresh bovine urine samples was monitored over the period 7 to 192 h after collection and ranged from 8.00 to 8.77. The pH of lyophilised urine samples ranged from 8.24 to 9.60. Amperometry was the most sensitive method among a range of electrochemical techniques in the detection of *p*-AP with a limit of detection (LOD) in urine of 1.0 μg mL^{-1} (10 μM) on a glassy carbon electrode.

Keywords: Electrochemical biosensors; Urine matrix effects; *p*-Aminophenol; Interferents

1. Introduction

The use of hormonal substances as animal growth promoters is prohibited for food safety reasons (EU Directive 88/146/EEC). Growth promoters are screened in a number of matrices such as kidney, fat, urine, and meats using different chemical and immunochemical [1] screening techniques followed by confirmatory techniques such as LC [2], GC–MS [3], LC–MS–MS [4], LC–ESI–MS [5]. All these techniques rely on random sampling, primarily at the point of slaughter, with specific tissue (muscle, liver, fat) or fluid (bile, urine) samples being taken to a centralised laboratory for analysis. The procedures involved in analysis can be relatively complex, and do not lend themselves to rapid measurement. These testing methods are not in widespread use due to the inherent cost involved, the ex situ nature of the analysis technique employed, the time lag involved (typically 24–36 h) and the relatively complex techniques used which require skilled laboratory personnel. Due to increasing enforcement of regulations governing the use of growth promoting agents, producers are rapidly changing the compounds used and the amounts administered, making detection much more difficult. Control of the use of growth promoters requires the development of a thorough screening programme [6]. The techniques used should be rapid, continuous and allow on the spot decisions to be made regarding the androgen residue status of an animal to assure the safety and integrity of the food supply.

The development of techniques which can reliably provide such assurances is difficult from a technical point of view, and probably, more importantly, in view of cost. A test kit which could provide semi-quantitative information, even if only on a restricted number of analytes, would be beneficial. Biosensors with an electrochemical transducer [7] may enable the implementation of such a screening program. As a sample matrix, blood is difficult to work with due to the large variable amount

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of red blood cells [8], and its high protein content. Sample processing of meat, faeces, and fats is also complicated. Urine contains less interference and is readily available at abattoirs and farms. As growth promoters are not generally electrochemically active, a recognition element such as an antibody coupled to an electrochemical transducer is required. The sensitivity of such an electrochemical ELISA technique depends on the ability of removing the effect of any species which interfere with electrochemical detection. It is necessary to determine the effect of each interfering species on the electrochemical response. In biological fluids, ascorbic acid, uric acid and xanthine are common electrochemical interferences. The composition of human urine [9] is well established. To our knowledge, the corresponding information on the composition of bovine urine is unknown. In this work, the concentrations of electrochemical interferences were determined and their effect on the electrochemical response of a model mediator, *p*-AP, was examined. We also describe methods of eliminating these interferences from biological samples and evaluated the best electrochemical technique for the detection of *p*-AP in bovine urine. The results of this work will then enable the development of an immunobiosensor for the detection of growth promoters in bovine urine.

2. Experimental

2.1. Reagents and chemicals

Lyophilised urine from 20 individual animals was obtained from the National Institute for Public Health and the Environment (RIVM) (Zeist, The Netherlands). 4-Aminophenol, urea, uric acid, 8-anilino-1-naphthalene sulphonic acid (ANS), bovine serum albumin (BSA), EDTA, xanthine, urea, uric acid and L-ascorbic acid were purchased from Sigma. $Cu(NO_3)_2$, KH_2PO_4, K_2HPO_4, uricase and Nafion® perfluorinated ion exchange resin were purchased from Aldrich. HCl and H_2SO_4 were obtained from Merck, uric acid test kits (catalogue no. 292) from Sigma. All aqueous solutions were prepared using purified water (18.2 MΩ) from an ElgaStat SPECTRUM system.

2.2. Apparatus

Electrochemical experiments were conducted using a three-electrode cell in which glassy carbon (GC, surface area 0.69 cm^2), platinum wire and Ag|AgCl were used as the working, counter and reference electrodes (CH Instruments), respectively. Electrochemical experiments were conducted using CHI 600 (CH Instruments) and PGSTAT 10 (Ecochemie) potentiostats. UV–vis spectra were obtained using a Shimadzu 1601 spectrophotometer. An Orion 420A pH meter was used to monitor the pH. Urine samples were lyophilised on an FTS Systems freeze-dryer.

2.3. Urine preparation

Urine specimens were collected from nine animals at the Lyons Research Farm (University College, Dublin) between 4 April 2003 and 7 May 2003. The study was performed in compliance with protocols approved by the Ethics Committee, University College Dublin, the Cruelty to Animals Act (Ireland, 1876), and the European Union Directive, 86/609/EC. All specimens were collected in 0.5 L containers (brown translucent material, light protected, with screw cap) on the farm and stored immediately at −20 °C in 70 mL containers. In addition, aliquouts (5 mL) of three fresh samples were lyophilised. The lyophilised urine samples obtained from RIVM were reconstituted by the addition of 5 mL of water.

2.4. Characterization of urine samples

Electrodes were polished successively with 1.0, 0.3 and 0.05 μm Al_2O_3 slurry on micro-cloth pads (Buehler), rinsed with distilled water and briefly sonicated. No additional electrolyte was added to the samples. Potentials are reported with respect to Ag/AgCl. Electrodes were modified by placing 10 μL of Nafion® solution on the electrode surface, followed by drying in air for 30 min.

The concentrations of ascorbic acid and xanthine were determined using HPLC. The chromatographic system was comprised of a Waters 484LC system utilising a reversed phase column (C18, 5 μm, 4.6 mm × 150 mm) (WAT 200662) and a UV detector. All experiments were performed at room temperature. In order to obtain good separation and reproducible results, the column was equilibrated for 4 days prior to use [10]. Levels of ascorbic acid were determined using a modification of the method described by Ross [11], with detection at 261 nm. The mobile phase was a mixture of K_2HPO_4 (5 mM) and H_2SO_4 (0.5 mM) pH 3 containing EDTA (200 mg/L), delivered at 0.35 mL/min. Urine samples were diluted 10 fold using 1% trichloroacetic acid to prevent sample decomposition and to adjust the pH to ca. 3.0. Stock solutions of ascorbic acid (1 mM) were prepared in 1% trichloroacetic acid. All solutions used were freshly prepared before each set of experiments.

The same column was used to determine the concentration of xanthine. Urine was diluted by a factor of 10 using 0.01 M phosphate buffer to pH 5.6. A mixture of K_2HPO_4 (0.01 M) and H_2SO_4 (0.5 mM) at pH 5.6 was used as the mobile phase and xanthine was detected at a wavelength of 267 nm. Standard xanthine solutions were prepared using the same procedure as with urine. Peaks due to ascorbic acid and xanthine were identified on the basis of the retention times of standards injected separately and by the addition of standards to urine.

Protein concentrations in urine were determined using the Bradford assay [12]. Solutions containing 0.01–0.1% (g/g) of BSA were used as standards. Uric acid test kits were used to quantify the concentration of uric acid in urine. Aliquots of urine (0.2 mL), glycine buffer (1.0 mL) and water (6.0 mL) were mixed and 3 mL of this mixture was placed in two different test tubes. 0.05 mL of uricase enzyme (0.2–0.4 U mL^{-1}) was added to one test tube and 0.05 mL water to the control solution. After 15 min, the absorbance was measured at 292 nm. The pH was measured in urine collected from the same animal at different time intervals from 7 to 192 h. During this time period, the animal had access to the same food area.