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A clinical assessment of direct electrochemical urate measurements

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Abstract

The response characteristics of various carbon substrates towards the direct oxidative measurement of urate and other key purine biomarkers have been compared. A novel carbon fibre laminate assembly has been proposed as an alternative substrate for the preparation of disposable sensing strips. The fabrication method is generic and readily transferable to a number of sensor applications. Its performance in the determination of urate within biofluids (serum and plasma) has been critically assessed. An inter-laboratory pilot study demonstrated the bioanalytical efficacy of the approach with the responses validated through comparison with the standard colorimetric (uricase/peroxidase) assay.

Keywords: Purines; Urate; Hypertension; Carbon fibre; Electrode; Electroanalysis; Diagnosis; Biomedical; Electrochemical; Composites

1. Introduction

Uric acid has long been recognised as a potentially significant diagnostic aid in the identification and treatment of cardiovascular disease [1], hypertension [2,3] and renal disease [4,5]. There is a substantial body of evidence to support the assumption that urate has an independent role in the pathogensis of these conditions [6,7]. Various animal models examining hypertension/renal function have demonstrated that urate is an important mediator of endothelial dysfunction, vascular disease, inflammation and nephrotoxicity [8–10]. It has since been shown that pharmacological intervention to reduce urate concentration in such instances can have a significant influence on the clinical outcome. The administration of allopurinol has been shown to lead to significant decreases in mortality rates in postoperative coronary contexts and effectively reversed endothelial dysfunction in heart failure subjects [11]. Improved cardiovascular mortality in the Losartan intervention for endpoint reduction in hypertension (LIFE) trials has also been attributed, in part, to the lowering of uric acid concentration [3]. Through being able to monitor the concentration of urate, it could be anticipated that predictive–preventative treatments could be employed at an early stage that could remove the potential for, or at least lessen the severity of, future complications.

The need for early identification and treatment lies in the fact that a number of studies have indicated that uric acid may be an important initiator of hypertension but may have little influence in the subsequent maintenance of the condition. Alterations to the intra-renal microvasculature initiated by hyperuricaemia appears to ultimately lead to a salt sensitive hypertensive state that becomes largely independent of urate concentration [8–10]. The administration of allopurinol has been shown to reduce both serum urate and blood pressure in adolescents and advances the case for the childhood screening of hyperuricaemia as a preventative measure [12,13]. Urate measurement currently requires referral to centralised laboratories, but if we were to consider identifying and countering the effects of primary hypertension then the development

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of new diagnostics that can facilitate regular, decentralised screening by health care visitors would be required.

Screen-printed electrodes (SPE) are widely used in biomedical testing and form the backbone of many of the commercial glucose sensors used in home diagnosis. Their use for the direct electrochemical detection of analytes within blood however is often hampered by poor resolution and sensitivity as a consequence of overlapping signals [14]. The approach taken here has been to examine the use of carbon fibre composite technology as a new disposable sensing substrate for the direct determination of uric acid in serum. Carbon fibre has been shown to provide a highly conductive substrate for use in a wide variety of electroanalytical investigations—particularly for in vivo microelectrode studies of neurotransmitters [15–17]. The key questions that need to be addressed in the present context relate to whether or not the material can exhibit the required sensitivity for purine analysis (principally urate) directly within serum and thereby serve as alternative to the colorimetric enzymatic method currently used.

The methodology is based around the use of thermal lamination methods where carbon fibre matting is sandwiched between insulating layers of a resin backed polyester film. A schematic outlining the key features of the detector design is shown in Fig. 1. The film is pre-punched so as to expose a defined working surface of the electrode with the laminate sheath providing a degree of mechanical strength but retains sufficient structural flexibility that allows easy manipulation of the strip design. The detection of urate is achieved through the reaction scheme shown in Fig. 1 and is reliant upon the direct oxidation of the urate (**I**) to the corresponding di-imine (**II**) [18,19]. This subsequently undergoes nucleophilic addition by two water molecules (**III**) leading to the allantoin (**IV**) the end product. The analytical signal however is derived solely from monitoring the current from the initial oxidation—the magnitude of which is proportional to the concentration of urate present within the sample matrix. The latter obviously relies upon the acquisition of a distinct signal with no contribution to the current occurring from other electroactive species (typically ascorbate). The present communication has sought to investigate the applicability of the sensing surface for use in analysis of clinical samples and to assess the efficacy of the technique when compared with conventional enzymatic protocols.

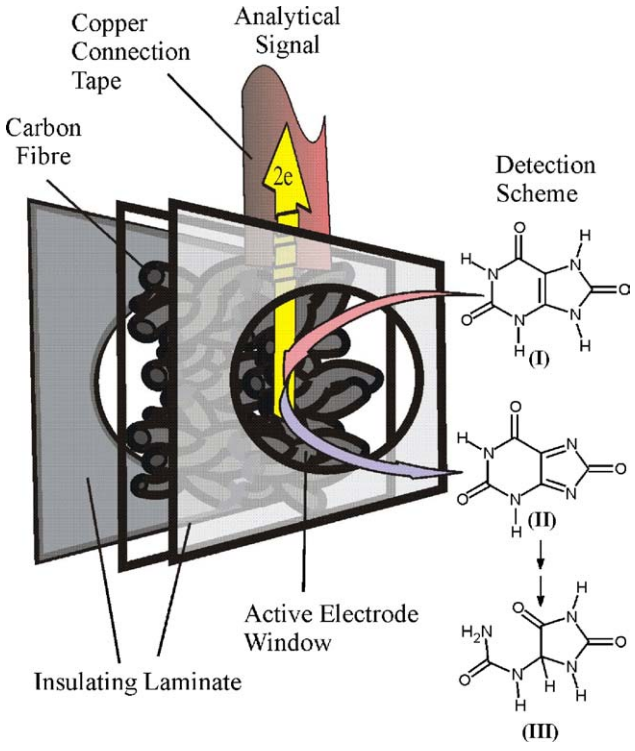

Fig. 1. Laminate design and detection mechanism.

2. Experimental details

2.1. Reagents and materials

All reagents were of the highest grade available and used without further purification. Stock solutions of uric acid (typically 10 mM) were prepared in 0.1 M NaOH. All other solutions were prepared using Britton–Robinson buffer (acetic, boric and phosphoric acids—each at a concentration of 0.04 M) adjusted to pH 7 through the addition of sodium hydroxide. Standard solutions were in prepared in deionised water from an Elgastat (Elga, UK) water system and refrigerated when not in use. Toray carbon fibre cloth was purchased from E-Tek Inc. (USA) and used as received. Lamination pouches (Rexel, UK) were a commercial stationary variety with a film thickness of 75 μm each side. Copper Shielding tape (100 μm thick, adhesive backed) was obtained from RS electronics.

2.2. Instrumentation

Electrochemical measurements were conducted using a μAutolab type III computer controlled potentiostat (Eco-Chemie, Utrecht, The Netherlands) using a three electrode configuration consisting of the carbon fibre assembly working electrode, a platinum wire counter electrode and a Ag | AgCl (3 M Cl^-) half cell reference electrode (BAS Technicol, UK). Square wave voltammetry was used throughout the electrochemical investigations (frequency: 25 Hz, step: 5 mV, amplitude: 20 mV).

2.3. Electrode construction

Laminated carbon fibre prototypes were prepared by thermally sandwiching carbon fibre sections (typically 8 mm × 8 mm) between sleeves of a pre-punched (5 mm dia window) resin-polyester lamination pouch using a commercially available laminator [20]. Electrical connection to the

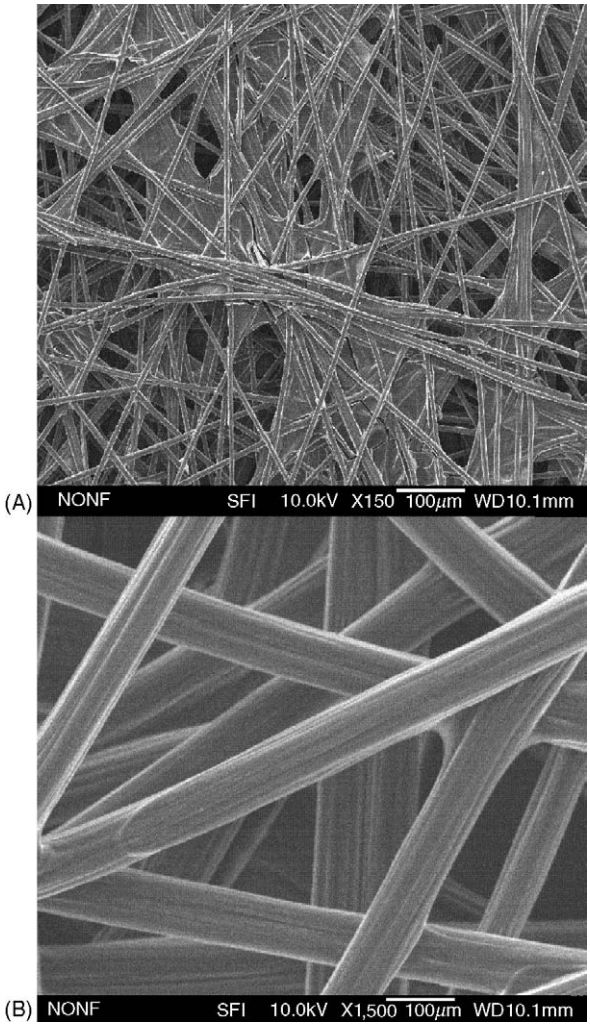

Fig. 2. Scanning electron micrographs of the carbon fibre framework.

carbon film was made through the presence of a strip of copper shielding tape. The electrodes were baked at 100 °C for 1 h in order to ensure the complete permeation of the resin between the fibres within the laminate. The mechanical integrity of the seal was chiefly assessed through scanning electron micrographic investigation of the morphology of the carbon fibre–resin–polyester interface. The lamination process was found to provide a coherent seal between the sensing fibre layer and the insulating polyester sheath with no solvent creep or de-lamination observed during extended immersion times (up to 20 min) in either aqueous solution or acetonitrile. The electrodes exhibited no degradation in response characteristics when repetitively cycled in buffer (pH 7) medium. This was also corroborated by the absence of any observable copper electrochemistry (assessed by cyclic voltammetry) that would have arisen had the permeation of solvent to the metallic conducting tract occurred. Scanning electron micrographs detailing the nature of the carbon fibre substrate and interlaced natured of the fibre network are detailed in Fig. 2A and B, respectively.

2.4. Clinical samples

A clinical trial involving two males and two females, ranging from 21 to 40 years of age was conducted. Samples of blood were obtained from the participants and analysed within 3 h of extraction. Blood was collected in gel permeation vaccutainers and then centrifuged at 3000 rpm for 10 min. The serum was withdrawn and used immediately for both electrochemical and spectroscopic analysis. Serum urate results were compared through inter-laboratory testing conducted within the biochemical laboratories at the Royal Surrey County Hospital NHS Trust. The UV–vis spectrometry technique uses a Bayer® assay (uricase/peroxidase/4-aminophenazone/*N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline, product no. B01-4131-01) with endpoint detection at 545 nm.

3. Results

The suitability of various carbon electrodes for detecting the purines was assessed predominantly using square wave voltammetry (SQWV) as it could be anticipated that the methodology would aid the resolution of discrete voltammetric signals [14]. The response of conventional bulk carbon (3 mm dia) electrode to different purine bases—urate (50 μM), xanthine (50 μM) and hypoxanthine (200 μM) in pH 7 buffer is shown in Fig. 3. The response to guanine (50 μM) and adenine (50 μM) were obtained separately and have been offset to avoid any ambiguities arising from peak overlap. Sharp, well defined and quantifiable processes are clearly observable. The expensive nature of glassy carbon combined with the difficulties in processing the material into a disposable format would clearly prevents its mainstream use in near patient testing contexts. The material should ideally possess similar sensitivity and resolution to that exhibited by the GC electrode but must be of a form that could be integrated into a disposable format.

The responses obtained at a carbon fibre laminate and at a commercial screen-printed carbon electrode towards the three main purines—urate, xanthine and hypoxanthine—are compared in Fig. 4A. The carbon fibre strip provides responses not unlike those observed with conventional glassy carbon (Fig. 3). The sharp peak resolution obtained at the carbon fibre strip however, contrasts that observed with the SPE and can be attributed to the composite nature of the latter (consisting of carbon particles, binders, etc.) [14,21–23]. The improvement in peak profiles arising from the homogeneous nature of the underlying carbon fibre provides a highly conductive track through which the current can pass. The heterogeneous make up of the SPE ink inevitably leads to slower electrode kinetics and hence larger overpotentials are invariably required to obtain a distinct and quantifiable current. This is seen in the shift in the purine peak positions observed between the carbon fibre laminate and SPE substrates with a separation of almost 150 mV. The SPE fares particularly

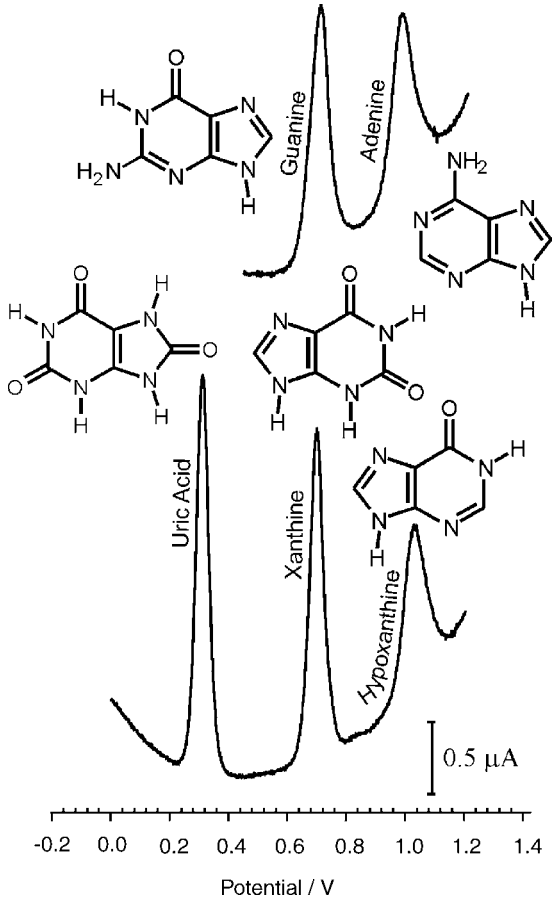

Fig. 3. Squarewave voltammograms detailing the response of a conventional glassy carbon electrode to key purine biomarkers in pH 7 buffer (UA = XA = Gu = Ad 50 μM, HX = 200 μM).

badly with hypoxanthine as the oxidation signal is almost indistinguishable from the solvent decomposition.

While well-defined responses can be obtained to each in isolation, complex media would be problematic and is highlighted in Fig. 3 by the potential overlap of xanthine with guanine and hypoxanthine with adenine. In contrast to most other purines, the determination of urate is generally free from the interference of other electroactive components even within complex biological media. Several catecholamines are also oxidised at low potentials and are potentially capable of overlap with the urate electrode process. These are normally present at low concentrations (i.e. dopamine <5 nM, epinephrine <5 nM, norepinephrine <5 μM) and would impart little to the overriding purine signal [24]. Ascorbic acid however, poses a considerable problem in that it is one of the few components present within biofluids that can be oxidised at potentials similar to that of urate and which can be present at relatively high concentrations (20–90 μM depending on dietary factor and physiological well being) [24].

Many strategies have been employed to counter the effects of ascorbate and facilitate the quantitative measurement of urate and include enzymes [25,26], polymeric coatings

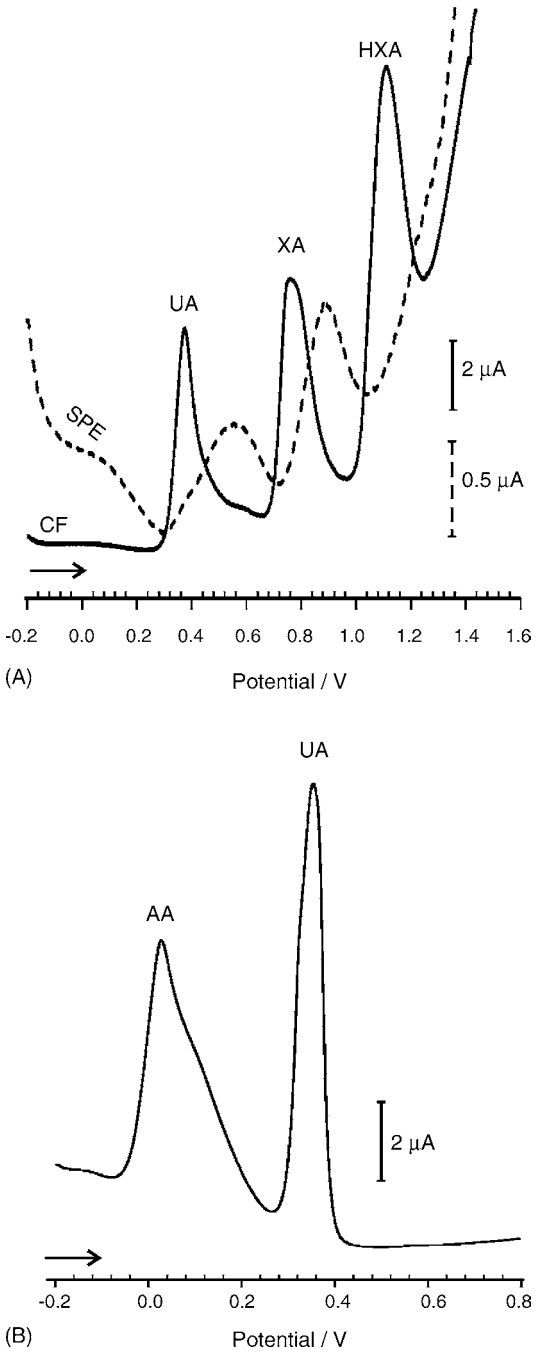

Fig. 4. (A) Squarewave voltammograms comparing the response to equimolar (300 μM, pH 7) urate, xanthine and hypoxanthine at a carbon fibre (solid line) and screen-printed (dashed line) electrodes. (B) Response to ascorbate/urate at a pre-anodised carbon fibre strip.

[27,28] and surface pretreatments [29,30]. The basis of electrolytic surface modification relates to the fact that carbon substrates tend to present a heterogeneous interface upon which carboxyl, phenolic and quinoid components are present. The populations of these species can be modified through the application of large anodic or cathodic potentials [29,30] and in doing so the electrode behaviour towards particular analytes altered. Pre-treating the carbon

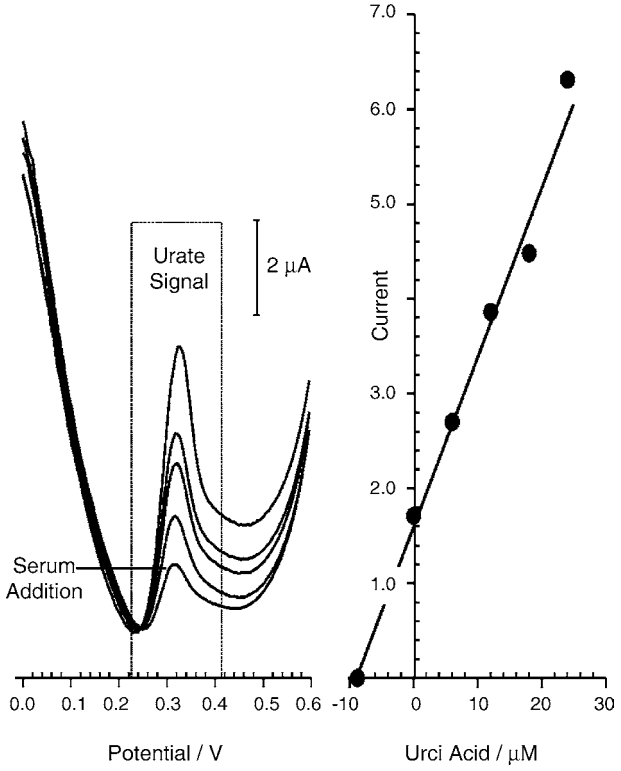

Fig. 5. Squarewave voltammograms detailing the response of pre-anodised laminate to serum urate (300 μL to 8 mL pH 7) and the subsequent additions of a standard urate solution (10 μL, 5 mM). Corresponding standard addition plot is include for comparison.

fibre substrate through anodising the surface (+2V, 0.1 M NaOH, 15 min) was found to yield considerable improvements in the response to both ascorbate and urate. Squarewave voltammograms detailing the responses of a pre-anodised carbon fibre electrode to ascorbate and urate are shown in Fig. 4B. The response to urate is preserved—as shown by comparison with Fig. 4B. The main enhancement however relates to the peak resolution (0.31 V) between the two components. Clear separation is possible and distinct baselines can be allocated from which to quantify urate.

The viability of the carbon laminate assembly for use in the analysis of complex biofluids was assessed through the determination of urate within plasma and serum. No pre-treatment of the sample was conducted beyond the centrifugal removal of the cellular components. In general, a 300 μL sample extract was placed within 8 mL of pH 7 buffer and squarewave voltammetry conducted as before. The voltammograms resulting from the addition of an initial serum sample and subsequent additions of a urate standard (10 μL, 5 mM) are shown in Fig. 5. The signal due to urate is distinct with the pre-anodisation of the fibre substrate providing an unambiguous baseline from which urate can be quantified. The process was repeated with plasma and near identical voltammograms obtained. In either case, the measurements were conducted in triplicate and provided an average urate concentration of 253 μM (%R.S.D. 6.4, $N = 3$) and 259 μM (%R.S.D. 9.8, $N = 3$) for the serum and plasma sample, respectively. The detection limit for the urate within pH 7 buffer at the pre-anodised fibre was 0.27 μM (based on $3S/N$) and provides a linear response over the range 0.3 μM to 50 μM (Ipa (μA) = 1.28 [UA/μM] −11.58, $N = 8$, $R^2 = 0.99$, based on a 5 mm diameter system). The intra reproducibility (same strip) was less than 5% but a higher degree of variability was observed (up to 25%) when comparing the responses of different strips. This can be attributed to the adhoc nature through which the strips were produced and inherent variations in the fibre network (Fig. 2A). Nevertheless, inter strip variability could be overcome through adoption of standard additions protocols and as can bee seen from the plasma and serum results the responses are within 10%.

Table 1
Comparison between the carbon fibre electrode and colorimetric methods for serum urate (300 μL) analysis

Subject	Laminate EC (urate/μM)	Colorimetric (urate/μM)
M1	309	290
M2	300	320
M3	328	360
M4	348	310
M5	339	320
M6	399	390
F1	415	460
F2	274	290

Analysis performed duplicate with a different strip used for each determination.

A mini clinical trial was subsequently conducted through which the electrochemical responses were compared with the standard colorimetric (bienzyme) method. The trial focused on the analysis of serum as this represents the medium through which standard clinical biochemistry procedures relate and allowed direct inter-laboratory comparison. The analysis was conducted as before but in duplicate and analysed using a single urate addition (10 μl, 5 mM). The results are summarised in Table 1 where it can be seen that the electrochemical method compares favourably with the independent laboratory results.

4. Conclusion

Modified carbon fibre laminates have been shown to facilitate the unambiguous analysis of urate within physiological fluids and the responses have been independently validated through inter-laboratory comparison. The material could be commercialised into a cheap, disposable format through the production of large area laminates within individual sensors arising through appropriate cutting of the composite sheet. As such, the approach could be a viable means through which point of care sensors could be produced. The electrode responses are superior to those obtained at commercial screen-printed electrodes with behaviour characteristic of a glassy carbon substrate but significantly less expensive. The approach taken is generic and sufficiently transferable for the

substrate and fabrication method to be considered for other sensing applications.

Acknowledgements

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