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Electrochemical detection and screening of bladder cancer recurrence using direct electrochemical analysis of urine: A non-invasive tool for diagnosis

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ABSTRACT

Although detection of urothelial cell carcinoma (or bladder cancer, BC) can be performed via cytological, molecular and genetic marker tests on urine, cystoscopy, an invasive technique, still remains as the gold methodology in clinical practice. It is presented a non-invasive method for detecting BC recurrence consisting of a direct electrochemical test in urine combining voltammetric data at gold and glassy carbon electrodes. The diagnosis is based on the ratio between characteristic voltammetric features recorded for tryptophan, serotonin and melatonin and other related metabolites. The method was tested by means of a clinical trial with 30 patients diagnosed of bladder cancer and a two control groups: 17 healthy volunteers and 15 patients diagnosed of other urinary pathologies and permitted detection of non-muscle invasive stages (pTa, pT1) and muscle-invasive stages (pT2 cancers) of BC.

1. Introduction

Due to the high prevalence and recurrence of bladder urothelial cell carcinoma, commonly known as 'bladder cancer' (BC), its detection in early stages is of crucial importance for its treatment [1,2]. The diagnosis of BC is usually achieved by means of biopsy obtained during endoscopy (or cystoscopy) study of the bladder, a technique involving the introduction of a rigid or flexible tube bearing a camera into the bladder through the urethra [3]. In order to dispose of non-invasive diagnostic criteria, several cytological [4], molecular [5,6,7] and genetic [8,9] marker tests have been developed. These correspond to the classic analytical methodology based on the determination of a label marker analyte [10]. As an alternative methodology, in vivo analysis during tissue resection by means of Raman spectroscopy [15] has been recently reported. These methodologies, however, suffer interference and matrix effects and/or often present problems of robustness, so that, in spite of intensive research, cystoscopy remains as the gold standard for diagnosing BC in the clinical practice [3,11–14].

An interesting analytical possibility consists of multi-analyte determination, as described for detecting breast cancer from total biochemical analysis of peripheral blood components [16]. This methodology has the intrinsic difficulty of multicomponent simultaneous detection in a very complex matrix, the relative high variability in the concentration of the different components due to alimentation, circadian cycles, etc. [11–14].

In this context, and aimed to develop a diagnostic tool of practical application in the clinical practice, we adopted a different, novel analytical strategy consisting of the use of a direct non-invasive voltammetric test combining gold and glassy carbon electrodes, based on the hypothesis that, in the absence of a specific marker compound, the occurrence of BC could be detected by appearance of specific variations in the relative composition of a set of electroactive urine components. The proposed method is inspired by the voltammetry of microparticles methodology [17–19], previously applied to detect hemolysis in blood [20] and screening antimalarials [21], and involves the record of voltammetric signatures of different metabolites. These include, in particular, tryptophan (Trp) and their metabolites, among others,

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serotonin (5-hydroxytryptamine, Ser), *N*-acetylserotonin (N-AcO-Ser), 3-indoleacetic acid (3-IAA), 5-indoleacetic acid (5-IAA) and melatonin as well as creatinine and catecholamines (dopamine, norepinephrine), all being electrochemically responsive. Because the bladder serves as a temporary urine reservoir, urine metabolites may provide potential candidates to be sensitive biomarkers [22,23]. Although the role of serotonin and melatonin in cancer is controversial [24], it has been reported that metastatic BCs are accompanied by low serotonin levels [25] whereas decreased melatonin levels [26] and increased levels of tryptophan and *N*-acetyltryptophan have been detected in BC patients [27,28]. Another by-product resulting from serotonin breaking, 5-IAA, has been reported as a cancer marker in urine [29].

It is pertinent to note, however, that urine is a multicomponent system containing a variety of metabolites. For instance, melatonin's major urinary metabolites, 6-sulfatoxymelatonin (aMT6s) and 6-hydroxy-melatonin glucuronide (GaMT), are highly stable products excreted in high amounts in the urine [30,31]. The disposal of a variety of sensitive and selective electrochemical sensors for determining Trp, Mel, Ser and/or 5-IAA [32–42] favors the use of electrochemical techniques for diagnosis. The above compounds plus uric acid, 5-Methoxytryptamine (5-MeO-Tra), *N*-acetylserotonin (N-AcO-Ser), creatinine, 5-methoxyindole-3-acetic acid (5-MeO-3-IAA), dopamine and norepinephrine were used as reference compounds for monitoring electrochemical response in artificial urine.

Application of electrochemical methods for diagnostic purposes, however, must face two general problems: a) the concentration of a given metabolite will depend, in general, of several biochemical factors, and b) the 'absolute' metabolite concentrations in urine vary depending on water intake (hydration status) and other factors, including circadian changes. In order to avoid the second problem, we adopted here an analytical strategy for electrochemical diagnosis differing from labeled detection, based, rather than on the determination of a threshold concentration for a marker analyte, on the variation of relative concentrations of different analytes, thus defining characteristic voltammetric features usable, via chemometric methods, for diagnosis.

For practical diagnostic purposes, and given the holistic-like strategy required to avoid sample pretreatment, the robustness was primed relative to selectivity. Accordingly, commercial, unmodified gold and glassy carbon electrodes were used as working electrodes. Given the different specific voltammetric response of the aforementioned electroactive metabolites on each one of these electrodes, they should provide two different sets of data able to be used as complementary analytical signals.

The proposed methodology was aimed to provide a practical tool for detecting BC recurrence and screening the different clinical stages of tumor progression using simple electrochemical tests in untreated urine spontaneously produced by the patients under 'ordinary' conditions of clinical practice. In this regard, it is pertinent to note that the primary aim of the analytical procedure was to insert it into a diagnostic process in which the urine of pre-diagnosed patients (i.e., that presenting symptoms leading to suspect the possibility of BC) was tested for screening those to be submitted to cystoscopy. As far as urine composition can be sensitive to diet, medication, circadian cycles, etc., the clinical trial was carried out with 30 patients (P-samples) diagnosed of BC in the non-muscle invasive (pTa, pT1 grades) and muscle-invasive stages (pT2 grade) and two control groups, formed by 17 healthy volunteers (H-samples), and 15 patients diagnosed of urinary pathologies different from BC (C-samples).

Both groups of C- and P-patients were polymedicated with antibiotics, analgesics and/or other drugs such as metotrexate. It has to be underlined that, although urine composition is particularly sensitive to BC [22,23], much of their tested electroactive components can act as markers of other types of cancer, so that the specificity of the analytical data has to be decided through a wider study.

2. Experimental

Urine samples were collected in hospital context between January 2016 and September 2017 from 30 patients (P01-P30) with confirmed BC, 17 healthy controls (H01-H17) and 15 patients undergoing screening for BC without pathological findings but displaying other urinary pathologies (C01-C15). The patients and C-controls were randomly selected from population performing routine bladder cancer screening and from population prior surgery. Healthy volunteers were also randomly selected from hospital and university population. According to the requirements of our ethics committee, all participants provided signed informed consent. Cancer diagnoses, which were graded into pTa (non-invasive papillary carcinoma), pT1 (Tumor invades subepithelial connective tissue) and pT2 (tumor muscle-invasive) types according to the WHO-1973/UICC-2017 classification, were confirmed by clinical, histological, and pathologic means.

For each participant, 2–5 mL of spontaneously produced urine were collected into sterilized tubes, stored at 5 °C, and processed within 6–10 h of collection. Complementary experiments were performed upon storing the sample urines at room temperature and processing it at times between 6 h and 7 days after collection. Voltammetric experiments were performed upon adapting a three-electrode tap to the urine-containing tube using a CH I 660c potentiostatic device (Cambria Scientific, Llwynhendy, Llanelli, Wales, UK). Platinum mesh and platinum wire were used as auxiliary and pseudo-reference electrodes, respectively. The working electrodes were glassy carbon (BAS, MF 2012, geometrical area 0.072 cm^2) and gold (BAS MF 2014, geometrical area 0.018 cm^2) electrodes which were activated by sequentially polishing with polishing cloths (BAS, West Lafayette, USA) with 1.0, 0.3, and 0.05 μm alumina in nanopure water followed by sonication in a glass vessel for 10 min. For testing repeatability, three independent voltammetric measurements were performed over each urine sample and replicating such experiments with three different working electrodes. Synthetic urine was prepared according to Laube et al. [43] using NaCl, KCl, $CaCl_2{\cdot}2H_2O$, Na_2SO_4, KH_2PO_4, NH_4Cl, and urea (all Merck reagents). Uric acid, L-Trp, 5-hydroxytryptamine, 5-Methoxytryptamine, *N*-acetylserotonin, 3-indoleacetic acid (3-IAA), 5-indoleacetic acid, dopamine and norepinephrine (Sigma-Aldrich) and creatinine, melatonin, 5-methoxyindole-3-acetic acid, and serotonin (Alfa Aesar) were used as reference compounds. Multivariate chemometric methods were applied to voltammetric data using MINITAB14 software package.

3. Results and discussion

3.1. General voltammetric pattern

Fig. 1 depicts square wave voltammogram at gold electrode of urine samples from a healthy volunteer, a patient of the control group and two patients diagnosed of BC at the pTaG2 and pTAG3 grades, respectively, where the potential scan was initiated at −1.25 V vs. Ag/AgCl in the positive direction. In these conditions, the voltammogram exhibits a prominent initial current associated to the hydrogen evolution reaction (HER) (notice the common profiles and reductive and oxidative signals in square wave voltammetry) followed by a series of oxidation signals between +0.40 and +1.25 V. On first examination, five signals at +0.45 (I), +0.70 (II), +0.85 shoulder, III), +1.00 (IV) and +1.20 V (V) can be distinguished. For each urine sample, these voltammetric signals remained essen-