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3,4-Dihydroxy-L-phenylalanine as a biomarker of oxidative damage in proteins: Improved detection using cloud-point extraction and HPLC

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ABSTRACT

Oxidized protein adducts are formed under conditions of oxidative stress and may represent a valuable biomarker for a variety of diseases which share this common aetiology. A suitable candidate biomarker for oxidized proteins is protein-bound 3,4-dihydroxyl-L-phenylalanine (L-DOPA), which is formed on 3′-hydroxylation of tyrosine residues by hydroxyl radicals. Existing methodologies to measure protein-bound L-DOPA employ lengthy acid hydrolysis steps (*ca.* 16 h) which may cause artifactual protein oxidation, followed by HPLC with detection based on the intrinsic fluorescence of L-DOPA. We report a novel method for the measurement of protein-bound L-DOPA which involves rapid hydrolysis followed by pre-column concentration of 6-aminoquinolyl-derivatives using cloud-point extraction. The derivatized material is resolved by reversed-phase HPLC in less than 30 min and has derivatization chemistry compatible with both UV and fluorescent detection, providing detection down to the femtomole level. The method provides identical results to those found with highly specific ELISA-based techniques and requires only basic instrumentation. The stability of the 6-aminoquinolyl-derivatives together with the fast and sensitive nature of the assay will be appealing to those who require large sample throughput.

1. Introduction

Oxidative modification of proteins is believed to play a major role in the pathogenesis of a variety of conditions, including cancer [1], Alzheimer's disease [2] and atherosclerosis [3]. Although virtually all classes of biological molecule undergo oxidation, the preeminence of proteins *in vivo* makes these a likely first target for oxidants such as hydroxyl radicals (HO$^{\cdot}$) [4], superoxide (HOO) [5] and reactive nitrogen species (RNS) [6]. The reaction of proteins with oxidants may bring about damage in a number of ways, including formation of protein hydroperoxides which mediate further oxidation [7], and formation of imines which lead to fragmentation of the polypeptide backbone [8]. Overall, the conformation and activity of the protein can be substantially altered, leading to disease progression. It therefore follows that the products of protein oxidation may serve as a useful metric of disease progression.

Although all twenty amino acids undergo oxidation modification, in order for the products of such reactions to be suitable biomarkers, they must be relatively stable and present at levels amenable to detection. As many of the products of protein oxidation are redox active and mediate further damage to the protein [9], measurement of these latter species may provide a more representative measure of protein oxidation *in vivo*. The aromatic amino acids (*viz.* phenylalanine, tyrosine, tryptophan and histidine) are particularly susceptible to oxidation and satisfy this criterion [10]. The oxidation of tyrosine mainly produces 3,4-dihydroxy-L-phenylalanine (L-DOPA) and a small quantity of its 2,4-isomer, ostensibly through a disproportionation reaction in anaerobic conditions [11], or by elimination of hydroperoxyl radical in the presence of oxygen [12]. L-DOPA is prone to further oxidation to produce a variety of o-benzoquinones which have a tendency to undergo Michael additions with thiols to produce 2-,5- and 6-cysteinyl-DOPA adducts which are involved in protein cross-linking [13,14]. L-DOPA itself is redox active and has sufficient reactivity (E° = 0.745 V) to bring about the reduction of copper(II) to copper(I) which may be associated with the concomitant production of superoxide anion which is a powerful oxidizing species [15,16]. The production of superoxide in this manner has been implicated in the formation of 8-oxo-7,8-dihydroxy-2′-deoxyguanosine, a key biomarker of oxidative DNA damage [17,18].

In considering the chemical and spectroscopic properties of our target analytes, we see that free tyrosine is marginally polar and has modest UV absorption (λ_{max} 278 nm; ε_{max} 1.10×10^3

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$M^{-1}\,cm^{-1}$) with conversion to L-DOPA being accompanied by a slight bathochromic shift (λ_{max} 280 nm; ε_{max} $2.71 \times 10^3\ M^{-1}\,cm^{-1}$); however, this is virtually indistinguishable under normal conditions. Accordingly, UV absorption alone cannot be utilized for measurement of these compounds and several alternative methods have been proposed. An early attempt by Arnow [19] was an adaptation of the xanthoproteic test for proteins, in which an alkaline nitrite/molybdate reagent reacts specifically with L-DOPA in the presence of tyrosine to produce an orange chromophore (λ_{max} 510 nm) and is sensitive down to *ca.* 2.5 nmol per sample. Later, Waite [20] reported measurement of L-DOPA by UV difference spectroscopy of (catechelo)borate complexes. This latter assay is less sensitive than the colorimetric procedure (*ca.* 10 nmol per sample) and is subject to interference from both tyrosine and tryptophan. Improved detection of L-DOPA can be obtained by employing HPLC with fluorescent detection (E_m 280 nm; E_x 320 nm) [21]. However, as this method relies on the intrinsic fluorescence of L-DOPA, results are subject to the usual variations associated with small changes in temperature and pH, as well as self-absorption at high concentrations of L-DOPA. Furthermore, the level of background fluorescence in serum-derived samples would be expected to be high without substantial sample extraction.

One potential solution to the issues of sensitivity and selectivity is to employ any of the amino acid derivatization agents, such as that reported by Waite, who derivatized peptidyl-L-DOPA using Edman's reagent [22]. However, in seeking to develop an assay for routine measurement of L-DOPA, we were keen to employ a derivatization procedure which would produce relatively stable derivatives which could be stored until analysis. A review of the common amino acid derivatization agents commercially available revealed 6-aminoquinolyl-*N*-hydroxyl-succinimidyl carbamate (AccQ) as an obvious choice. AccQ reacts with primary and secondary amines to yield 6-aminoquinolyl-derivatives and *N*-hydroxysuccinimide (Scheme 1). This reaction is rapid and produces derivatives which are stable and respond by fluorescence (E_m 348 nm; E_x 395 nm), UV absorption (λ_{max} 254 nm) or electrochemical detection [23,24].

In this work, we report an improved HPLC-based method for the determination of L-DOPA in both purified proteins and human serum. By employing a rapid, anaerobic acid hydrolysis step, we have minimized artifactual oxidation of amino acid residues and have used AccQ reagent to produce 6-aminoquinolyl-derivatives which are resolved by reverse-phase HPLC. The sensitivity of the assay has been significantly increased by incorporating a concentration step utilizing cloud-point extraction. In this procedure, hydrophobic compounds (in this case, mainly the 6-aminoquinolyl derivatives) are partitioned in a small, surfactant-rich layer, which on addition of concentrated salt solution becomes turbid (the 'cloud-point'), permitting separation from underivatized components by centrifugation [25,26]. This rapid, cost-effective method is ideal for large-scale studies where high sample throughput is a desired and is sufficiently versatile to permit measurement on basic HPLC systems without a significant decrease in sensitivity.

2. Materials and methods

2.1. Preparation of reagents

All chemicals were of at least analytical grade and purchased from Sigma–Aldrich (Dorset, UK) unless otherwise stated. Aqueous solutions were prepared using Milli-Q double-deionized water (resistance > 18 $m\Omega/cm^2$) (Millipore, Bedford, MA, USA) stored over Chelex-100 resin to eliminate adventitial transition metal ions. Protein (fatty acid free bovine serum albumin, BSA) was used as supplied or oxidized (0.06 mg) by AAPH (10 mg/mL) at 50 °C for 3 h in sodium phosphate buffer (10 mmol/L, pH 7.4). The oxidized proteins were stored under helium at −20 °C until required.

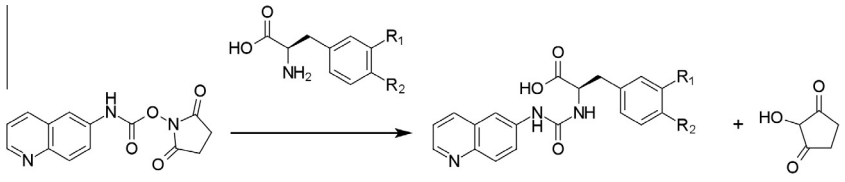
2.2. Protein purification and hydrolysis

Fasting peripheral venous blood samples were collected from healthy volunteers by standard venipuncture of a prominent vein in the antecubital fossa. Collected blood was transferred into K_3-EDTA tubes (Vacuette) containing 100 μmol/L BHT and plasma recovered by low-speed centrifugation. Plasma (700 μL) was combined with 100 μL trichloroacetic acid (50%) and 50 μL sodium deoxycholate (0.3%) and the protein precipitate recovered by centrifugation at 6000 rpm for 10 min. The precipitate was washed three times (alternately) with 800 μL of acetone and 800 μL diethyl ether and dried under vacuum.

Proteins (oxidized BSA and purified human plasma proteins) were hydrolyzed according to the method of Tsugita et al. with minor modification [27]. Briefly, aliquots (500 μL) of protein (0.1 mg/mL) were transferred to gas-tight Mininert® vials (Supelco, PA, USA) and combined with concentrated hydrochloric acid/trifluoroacetic acid (2:1 v/v) containing phenol (1%) and mercaptoacetic acid (5%). The tube was sealed, evacuated of air and the solution deoxygenated by bubbling with helium for 30 s; this process was repeated a further three times. Samples were heated at 160 °C for 25 min, dried and reconstituted immediately before use in 1 mL ddH_2O.

2.3. Derivatization and concentration of hydrolysates

Hydrolyzed samples (1 mL) were combined with 70 μL borate buffer (0.2 mol/L, pH 8.8), 40 μL AccQ reagent (Waters, Milford, MA, USA) which was used neat (for fluorescence detection) and also at a 1/10 dilution (for UV detection). Samples were vortexed for 30 s and heated at 70 °C for 30 min. As preliminary results for UV detection were poor (see Results), a pre-column concentration step was added in the form of cloud-point extraction. To derivatized samples, 200 μL of Triton X-114 and 300 μL of ammonium sulfate (3 mol/L) were added. The mixture was gently inverted to allow the cloud point to form and complete separation was achieved by centrifugation at 6000 rpm for 10 min. The upper aqueous phase was discarded and the volume of the lower phase (*ca.* 100 μL) adjusted to 200 μL with ddH_2O, vortexing briefly to ensure a homogeneous solution. L-DOPA standards (0.005–100 nmol/L) and a set of twenty amino acids standards were also prepared.



Scheme 1. Derivitization of L-DOPA. Tyr $R_1 = 1$; $R_2 = OH$; L-DOPA $R_1 = R_2 = OH$.