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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.

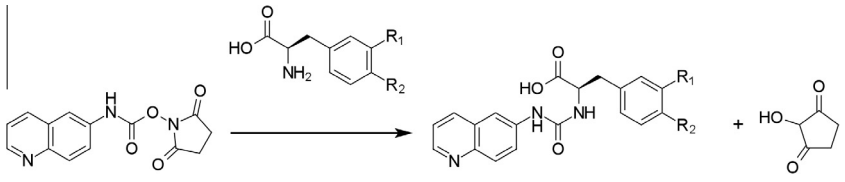
2.2. Protein purification and hydrolysis

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 $R_1 = R_2 = OH$.

2.4. HPLC of derivatized amino acids

Derivatized amino acids (20 μL sample injection) were resolved on a Phenomenex C_{18} column (3.9 × 300 mm; 5 μm) using a Perkin-Elmer Series 200 HPLC system. A gradient of solvent A (Waters AccQ.Tag™ Eluent A) in solvent B (60% acetonitrile; Ridel-de Haen, Steinheim, Germany) was used at a flow rate of 1 mL/min. The gradient was programmed as follows: 0–5 min, 0–6% B; 5–6.5 min, 6–10% B; 6.5–11 min, 10–33% B; 11–13 min, isocratic 33% B. A wash-out sequence of 13–14 min, 33–100% B; 14–15 min, isocratic 100% B; 15–16 min, 100–0% B completed a sample run. The eluent was monitored by either diode array detection at λ_{max} 254 nm (Method A) or fluorescence (E_x 395 nm; E_m 348 nm) (Method B). For comparison, HPLC employing detection by intrinsic fluorescence of tyrosine/L-DOPA (E_x 280 nm; E_m 320 nm) (Method C) was also employed as previously reported [16].

2.5. Miscellaneous methods

Independent measures of protein oxidation were used at various stages in the development of the analytical procedure. Total protein carbonyls were determined spectrophotometrically as their 2,4-dinitrophenylhydrazone derivatives [28]. The concentration of L-tyrosine and L-DOPA were determined by commercially available enzyme-linked immunosorbent assays (tyrosine: Immundiagnostik AG, K7015; L-DOPA: BioSource ELISA kit, MBS162177).

2.6. Method validation and statistical analysis

Routine descriptive statistics were generated from analysis of data using Microsoft Excel (2007) and results expressed as mean ± standard deviation. Non-parametrically distributed results were analyzed by the Mann Whitney *U*-test. A comparison of the methods was achieved by two-way analysis of variance (ANOVA). Both statistical tests were performed using the Statistics Package for Social Sciences (SPSS) Version 17.0 for Windows. $P < 0.05$ was considered as statistically significant.

3. Results and discussion

3.1. Preparation of BSA sample material

To provide readily available source of sample material for the development and characterization of the assay, BSA was oxidized by alkylperoxyl radicals generated by the thermal decomposition of the azo-initiator AAPH [29]. The levels of protein carbonyls were assessed to verify adequate oxidation of the protein, and provided results in keeping with those available in the literature (4.9 ± 0.2 nmol/mg BSA) [30]. The levels of L-tyrosine (30.9 ± 2.3 nmol/nmol BSA) and L-DOPA (39.6 ± 2.3 pmol/nmol BSA) were determined by ELISA and provided an estimate of the expected concentrations of these analytes.

3.2. Reversed-phase HPLC of derivatized amino acids and L-DOPA

A set of twenty standard amino acids plus L-DOPA (2.5 mol/L) were derivatized and subjected to HPLC initially using conditions recommended by the manufacturer [31]. The modification of these conditions reported in the Experimental section afforded good resolution of the amino acids peaks, with peak heights in keeping with the derivatization chemistry. The position of the L-DOPA peak was predicted to be in advance of tyrosine, based on the presence of an additional –OH group which increases the polarity of the molecule, resulting in L-DOPA eluting before tyrosine and the remaining (non-polar) amino acids. Results (Fig. 1) are in keeping with this prediction, with 6-aminoquinolyl-tyrosine and -L-DOPA eluting mid-profile with average retention times (R_t) of 7.8 ± 0.02 and 7.5 ± 0.09 min, respectively ($n = 12$). These retention times matched those obtained for a simple sample containing only L-DOPA and L-tyrosine (Fig. 1, inset). The resolution of the amino acid derivatives was virtually identical for both detection modes (UV, Method A and fluorescence, Method B), as expected. The large initial peak ($R_t \approx 2$ min) was the co-product of the derivatization reaction, N-hydroxysuccinimide, though it appeared that neither the presence of this additional peak nor those of any of the twenty common amino acids interfered with the identification of the target analyte.

3.3. Analytical performance

The measurement of L-DOPA was found to be completely linear over the calibration range employed for both Method A and Method B (0.005–100 nmol/L; correlation coefficient of $r = 0.9998$) with an identical relative standard deviation (RSD) of 2.7% for both methods. This made the routine determination of L-DOPA straightforward as a non-weighted linear calibration function (peak response *vs*. concentration) could be used. The limits of detection (LOD) and limits of quantification (LOQ) were calculated from the standard deviation of blank samples and the gradient of the calibration line, assuming a signal-to-noise ratio of 3 and 10, respectively (Table 1). The repeatability and reproducibility of the assay, taken as intra-day and inter-day RSD, was similar for both methods (Table 1). Overall, the performance characteristics for Methods A and B were superior to those found for the assay employing intrinsic fluorescence (Method C), particularly with regard to the LOQ and inter-day CV.

In establishing our assay, we selected detection by either UV or fluorescence as at least one of these detection modes will be available on even the most basic HPLC instrument and is therefore accessible to a wide-range of laboratories. We did not investigate the use of electrochemical detection as this is generally comparable to fluorescence detection in terms of sensitivity, as shown by Pappa-Louisi et al. [32] and would therefore offer no analytical advantage. We found that the anticipated detection method had an impact on the derivatization procedure employed, as preliminary experiments utilizing neat AccQ reagent and UV detection demonstrated poorer resolution of peaks, due to the dominance of the *N*-hydroxysuccinimide peak. It was found that when using UV detection, a 1/10 dilution of AccQ reagent could be used with no observable effect on the derivatization reaction, yet substantially improving the resolution of peaks. The neat reagent was required for optimum detection by fluorescence down to the pmol/sample level.

3.4. Impact of cloud-point extraction

When dealing with samples of a more complex nature (BSA or human plasma), it was found that detection of tyrosine and L-DOPA was limited to the submicromolar range for UV detection and the nanomolar range for fluorescent detection, and thus did not represent a particularly significant improvement. However, when samples were pre-concentrated using cloud-point extraction, the limit of detection was shifted by an order of magnitude for both detection methods and enabled detection of L-DOPA in unoxidised BSA using UV detection (Table 2).

The effect of cloud-point extraction is twofold. Firstly, as Triton-X114 is amphiphilic, micelles are formed in aqueous solution with a highly hydrophobic core. The non-polar components of the reaction mixture (mainly AccQ-derivatives) migrate from the aqueous buffer solution to the hydrophobic core, separating them from

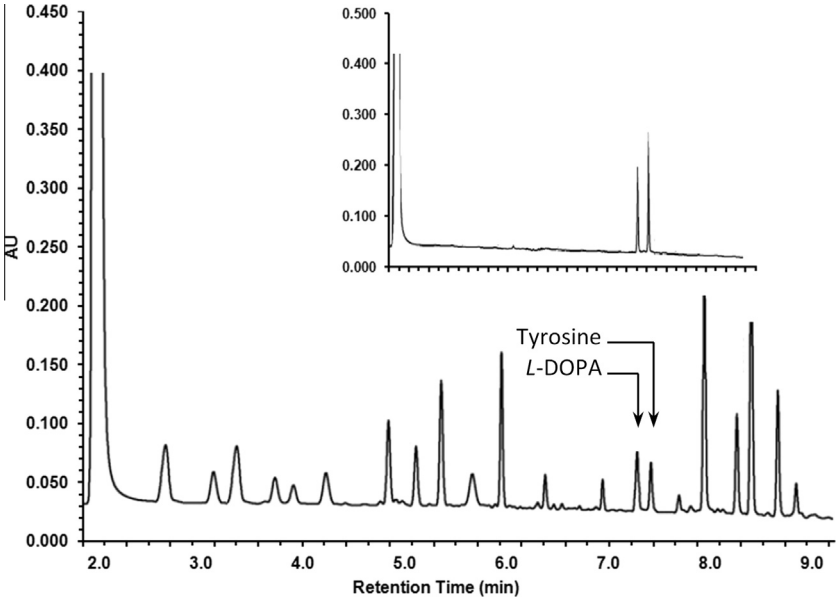


Fig. 1. Representative chromatogram for separation of 6-aminoquinoyl amino acid derivatives. L-DOPA eluted between serine and tyrosine, which is consistent with the polarity of the molecule under the solvent conditions employed.

Table 1
Performance characteristics for measurement of L-DOPA.

	Method A[a]	Method B[b]	Method C[c]
LOD (20 μL injection)	0.42 ± 0.01 pmol	2.10 ± 0.11 fmol	0.15 ± 0.03 nmol
LOQ (20 μL injection)	1.41 ± 0.05 pmol	7.06 ± 0.16 fmol	1.08 ± 0.07 nmol
Recovery	98.4 ± 1.2%	97.8 ± 1.9%	119.2 ± 1.5%
Intra-day CV	1.1%	1.8%	3.9%
Inter-day CV	2.3%	3.4%	10.8%

[a] Method A, UV absorption (λ_{max} 254 nm).
[b] Method B, fluorescent detection (E_x 395 nm; E_m 348 nm).
[c] Method C, HPLC employing intrinsic fluorescence (included for comparison).

Table 2
Effect of cloud-point extraction on measurement of tyrosine and L-DOPA.

	Method A	Method B
Without cloud-point extraction		
Tyrosine (nmol/nmol BSA)[a]	47.12 ± 1.43	41.49 ± 3.22
L-DOPA (pmol/nmol BSA)[b]	–	2.67 ± 1.38
With cloud-point extraction		
Tyrosine (nmol/nmol BSA)	34.13 ± 2.92	33.80 ± 2.16
L-DOPA (pmol/nmol BSA)	1.59 ± 0.16	1.57 ± 0.19

[a] Mean tyrosine concentration (by ELISA) for same samples = 30.9 ± 2.3 nmol/nmol BSA.
[b] Mean L-DOPA concentration (by ELISA) for same samples = 1.62 ± 0.3 pmol/nmol BSA.

potential hydrophilic interferences which may have UV or fluorescent properties. Secondly, as the AccQ-derivatives are transferred from a larger volume (the aqueous phase) to a smaller volume in an equilibrium-dependent manner, the desired components are concentrated by a factor easily determined from experimental data [33]. In addition to improving the sensitivity of the assay, cloud-point extraction substantially reduced the peak due to *N*-hydroxysuccinimide and completely eliminated a peak believed to be due to free phenol (used in the hydrolysis step). Therefore, the incorporation of this additional step is worthwhile, especially if the UV detection mode is used.

3.5. Effect of protein hydrolysis on L-DOPA

Owing to the labile nature of tyrosine, it is possible that a portion of the tyrosine residues in a given sample may undergo oxidation during the acid hydrolysis step. Such artifactual alterations in L-DOPA expression were assessed by determining the amount of tyrosine present in a BSA sample by ELISA and then subjecting the sample to our hydrolysis procedure and re-assessing the levels of tyrosine present by ELISA and HPLC. The effect of acid hydrolysis on the levels of protein-bound L-DOPA was assessed in a similar fashion. Results (Table 3) demonstrated no significant decrease in the amount of tyrosine detected, implying that virtually no tyrosine residues had undergone conversion to L-DOPA as a consequence of acid hydrolysis. There was a slightly larger decrease in the levels of L-DOPA following hydrolysis, most likely due to further oxidation of L-DOPA to a variety of o-benzoquinone intermediates. However, this decrease was not statistically significant ($P > 0.05$, $n = 12$) and should therefore not present any major underestimation of L-DOPA.

Taking these findings into consideration, the hydrolysis procedure presented in this work seems preferable to other methods, not only because it requires a much shorter incubation time (25 min *vs.* 16 h), but also because it is performed in an inert atmosphere in the presence of mild reducing agents. These precautions were worthwhile, as BSA hydrolyzed by a traditional method (6 M

Table 3
Levels of tyrosine and L-DOPA before and after protein hydrolysis.

	Tyrosine[a]		L-DOPA[b]	
	Before	After	Before	After
Method A	32.14 ± 1.9	31.07 ± 1.3	1.64 ± 0.11	1.49 ± 0.23
Method B	33.23 ± 0.6	32.44 ± 2.1	1.71 ± 0.31	1.68 ± 0.13
ELISA	33.98 ± 3.7	34.63 ± 6.1	1.75 ± 1.99	1.73 ± 2.93

[a] nmol tyrosine/nmol BSA.
[b] pmol L-DOPA/nmol BSA.

HCl for 16 h at 110 °C) showed substantial over-recovery of L-DOPA (142.0 ± 2.3%; $P < 0.05$; $n = 3$).

3.6. Application to human plasma samples

To validate our method for a more complex sample matrix, plasma was obtained from twenty volunteers and subjected to our analytical method as well as independent ELISA-based methods. Results (Fig. 2) show a strong correlation between the two methods, with an average plasma L-DOPA concentration of 918 ± 4.8 μmol/mol tyrosine, which is in keeping with those found in the literature [34]. Furthermore, given the widely-accepted specificity of antibody-based techniques such as ELISA, the correlation between these two methods is encouraging. As the primary application of this assay is for the measurement of L-DOPA in human plasma samples, and important consideration is the stability of the 6-aminoquinolyl derivatives, should they be prepared and analyzed *en masse* at a later date. We assessed the stability of a pooled 6-aminoquinolyl derivative over a thirty-day period stored at 4 °C. The results (Fig. 3) demonstrate that the L-DOPA derivative is stable within an acceptable margin; the corresponding 95% confidence interval of the data in Fig. 3 is ± 1.45 μmol L-DOPA/mol tyrosine.

Considerable emphasis has been placed on identification of suitable biomarkers of oxidative stress. In this work, we report a rapid, reliable and sensitive assay for protein-bound L-DOPA based on the separation of 6-aminoquinolyl derivatives by reversed-phase HPLC. The increased sensitivity of the assay is a combined result of the derivatization chemistry and the application of a pre-column cloud-point extraction procedure. This method outperforms the frequently used HPLC method employing detection of intrinsic fluorescence in terms of sensitivity and precision, and on the basis of comparison to ELISA data, exhibits notable specificity. This method will be particularly appealing to those laboratories equipped with minimal or limited instrumentation, with the method employing UV detection still offering adequate sensitivity for routine measurements.

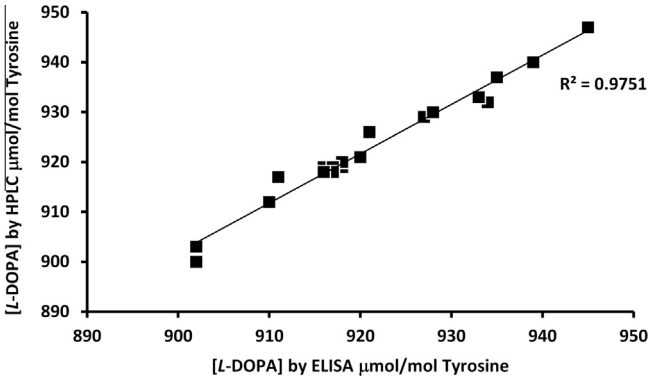


Fig. 2. Correlation of plasma L-DOPA measurements made by ELISA and HPLC.

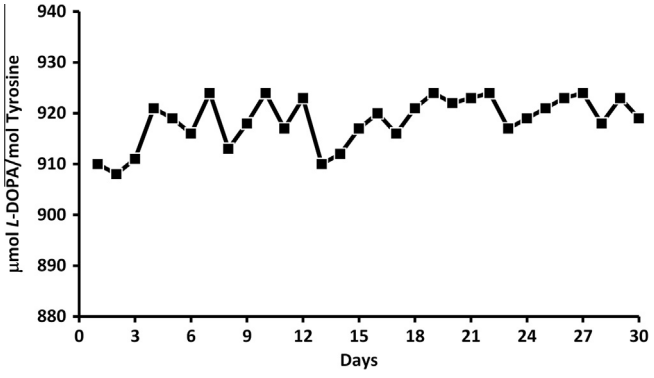


Fig. 3. Stability of 6-aminoquinolyl-L-DOPA over a thirty-day period. The mean of these data was 910.57 ± 3.10 μmol L-DOPA/mol tyrosine.

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