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Electrochemical activity of 6-aminoquinolyl urea derivatives of amino acids and peptides. Application to high-performance liquid chromatography with electrochemical detection

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Abstract

6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate (6-AQC) is a reagent used to increase the detection-sensitivity of amino acids and peptides in high-performance liquid chromatography with fluorescence detection. In this paper, the electrochemical characteristics of the derivatives of 6-AQC are described. Electrochemical detection of 6-AQC amino acids and peptide derivatives following reverse-phase HPLC are also reported. The response linearity of the derivatives on an amperometric detector was studied in the range of 5 pmol (0.5 μM) to 2500 pmol (250 μM). Approximately 2.5 pmol of the amino acid derivatives could be detected. The quantitative results of amino acids in plasma and a bovine serum albumin hydrolysate agreed well with values reported in the literature.

Keywords: Derivatization, LC; Amino acids, 6-aminoquinolyl urea derivatives; Peptides; 6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate

1. Introduction

Amino acid (AA) and peptide analysis with reversed-phase HPLC has become an important technique within the past decade. Because of the lack of intrinsically-sensitive chromophores and fluorophores, the demand for sensitive and selective methods for detection of AAs and peptides has led to the development of more sensitive derivatizing reagents. For AAs, the derivatization schemes include those based on *ortho*-phthalaldehyde (OPA) [1–3], 9-fluorenylmethyl chloroformate (9-FMOC) [4,5], 1-dimethylaminonaphthalene-5-sulfonyl chloride (Dansyl-Cl) [6,7], 4-dimethylaminoazobenzenesulfonyl chloride (Dabsyl-Cl) [8,9], 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) [10,11], phenylisothiocyanate (PITC) [12,13], fluorescamine [14,15] and naphthalene-2,3-dicarboxaldehyde (NDA) [16–18]. Precolumn derivatization of AAs with these reagents not only enhances detection sensitivity, but also improves the retention behavior

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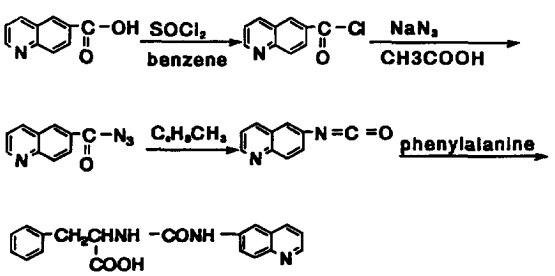
of AAs. Recently, a fluorescence detection (FL) derivatizing reagent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (6-AQC) has become popular in the analysis of AAs and peptides [19–21]. This is due to the stability of its derivatives, excellent derivative yields (approx. 100%) and no interference from the only major FL by-product, 6-aminoquinoline (6-AQ).

Electrochemical (EC) detection provides a sensitive and specific method for detecting compounds possessing intrinsic EC activity. EC detection has been used already for the determination of AAs, such as tryptophan (Trp), tyrosine (Tyr) and cysteine (Cys), that contain EC active groups [22,23]. However, most AAs are not intrinsically EC active and cannot be determined directly with an EC detector. Derivatization with specific reagents provides an opportunity to make the derivatives of AAs and peptides EC active. It has been demonstrated that some derivative types, e.g., OPA and NDA, containing the iso-indole group, are EC active and these have been determined with EC detection [24–28].

The structure of 6-AQC-derivatized AAs contain an aromatic amine group, i.e., aminoquinoline, Scheme 1. In view of the EC activity of aromatic



Scheme I. Synthesis of 6-AQC phenylalanine

amines (e.g., 2-aminoquinoline, aniline), 6-AQC derivatives of AAs might be electrochemically determined. In this paper, the EC properties of 6-AQC urea derivatives of AAs and peptides have been studied. The rationale for studying this new derivatizing reagent by HPLC with EC (LCEC) has been its well-characterized UV–FL detector properties and its lack of any demonstration of EC capabilities. Though other amino acid and peptide derivatizing reagents, such as OPA and NDA, exist and have UV–FL and EC detection properties, the 6-AQC reagent offers an ease of reaction chemistry, absence of FL properties of the hydrolysis products and sample preparation and clean-up steps that are perhaps easier than with other, related derivatizing reagents [12,19–21,30]. Because of the current, intense interest in applying the 6-AQC reagent to many amine-containing chemicals, drugs, peptides, and even proteins, it appeared opportune to provide analysts with additional detector approaches, such as EC [20,34]. It was not intended that 6-AQC would be a replacement derivatizing reagent to OPA or NDA for LCEC of amine-containing compounds, such as AAs or peptides [24–28]. Using 6-AQC, analyses of AAs in plasma and a protein hydrosylate and of peptides in an enzymatically digested protein, were performed with LCEC.

2. Materials and methods

2.1. Chemicals

AAs, peptides, proteins, human and bovine plasma, and trypsin-L-1-tosylamido-2-phenylethyl chloromethyl ketone (trypsin–TPCK) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Derivatizing reagent, 6-AQC (Waters AccQ.^{TM} Fluor Reagent) and borate buffer (pH 8.8) were obtained as a kit from Waters Corporation (Milford, MA, USA). Phosphoric acid and sodium dihydrogen phosphate (monobasic) were obtained from Fisher Scientific Company (Fair Lawn, NJ, USA). Quinoline-6-carboxylic acid was purchased from ICN Biomedicals (Costa Mesa, CA, USA). HPLC-grade acetonitrile (ACN) and water (H_2O) were generously donated by EM Science (Gibbstown, NJ, USA).

2.2. Synthesis and characterization of 6-AQC-phenylalanine standard

There is no indication in the literature that this particular compound has been synthesized or characterized previously. A 0.76-g (4.4 mmol) amount of quinoline-6-carboxylic acid, 30 ml benzene, 0.6 ml triethylamine (TEA) and 1.28 ml thionyl chloride ($SOCl_2$) were added to a 50-ml round-bottomed flask and stirred at 65–75°C for 1 h. The solvent was rotoevaporated and 8.9 ml acetic acid (AcOH), with 286 mg sodium azide (NaN_3), was added. The

solution was stirred at room temperature (rt) for 2 h and was then added to 100 ml H_2O. 16 ml TEA was added, the mixture was stirred and filtered. The solid was washed using 400 ml H_2O, dissolved in a small amount of AcOH and filtered. 150 ml H_2O was added to the filtrate and this was again filtered. The solid was dried overnight under vacuum, and light brown crystals were obtained. The crystals were dissolved in 80 ml toluene, and to this solution, 1 g of L-phenylalanine (Phe) was added. The mixture was stirred at 105–115°C for 30 min, then at 90°C for 30 min, at 60–70°C for 2 h and at rt for 1 h. The reaction slurry was filtered and the product was recrystallized from dimethyl formamide (DMF) and H_2O, as off-brown crystals, m.p. 207–208°C (decomposed). The synthesis is shown in Scheme 1.

Analysis Calculated for $C_{19}H_{17}O_3N_3$: %C, 68.06; %H, 5.11; %N, 12.54. Found: %C, 68.01; %H, 5.17; %N, 12.73.

^{1}H NMR 300 MHz (Varian XL300 FT Spectrometer, Varian Inc., Palo Alto, CA, USA) (d_6-DMSO) δ 3.05 (2H), 4.50 (1H), 6.48 (1H), 7.25 (5H), 7.42 (1H), 7.58 (1H), 7.88 (1H), 8.07 (1H), 8.20 (1H), 8.70 (1H), 9.05 (1H). Chemical-ionization mass spectrometry (Finnigan MAT Model 90, Finnigan MAT, San Jose, CA, USA) *m/z* (relative intensity), M+1 336.1 (3.39), 319 (25.95), 318 (100), 171 (54.28), 145 (49.62), 91 (27.97).

2.3. Derivatization of AAs and peptides

The literature procedure [19] was followed for the derivatization of AAs and small peptides with 6-AQC reagent. A 10-μl aliquot of an AA- or peptide-containing solution was placed in a 6×50 mm glass tube. A 70-μl volume of borate buffer (0.2 *M*, pH 8.8) was added and the mixture was vortexed. A 20-μl volume of a solution containing the 6-AQC reagent (3 mg/ml dissolved in ACN) was added and the solution was vortexed quickly to ensure complete derivatization of all amines. The samples were then heated for 10 min at 50°C to convert any di-derivatized product into the mono-derivatized product of Tyr. A serial dilution of the initially derivatized solution (high concentration) was performed when necessary, before HPLC injection, e.g., in the derivation of calibration plots, linearity and detection limits.

2.4. Acid hydrolysis of protein

Samples containing about 25 μg of bovine serum albumin (BSA) were vacuum-dried in 6×50 mm tubes using a Pico·Tag® workstation. Hydrolysis tubes were placed in larger vials and 200 μl of 6 *M* HCl and one crystal of phenol were added to the outer vials. The tubes were sealed under vacuum and heated at 153°C for 1 h. The samples were reconstituted with 100 μl of 20 m*M* HCl and 10μl of this solution was derivatized.

2.5. Trypsin digestion of cytochrome c

A 2 mg/ml solution of cytochrome *c* (cyt *c*, from horse heart) was prepared in 100 m*M* ammonium bicarbonate buffer, pH 8. Trypsin-TPCK was dissolved in the same buffer at a concentration of 0.1 mg/ml. To 0.5 ml of cyt *c* solution, 0.5 ml of trypsin solution was added. Digestion took place at 37°C for 24 h and was then terminated by heating the digestion solution at 100°C for 5 min [29]. The digested solution was lyophilized and reconstituted with 1.0 ml H_2O and 10 μl of the solution was derivatized.

2.6. Deproteinization of plasma

To a 1.2-ml centrifuge tube, 0.5 ml of plasma (human or bovine) and 0.5 ml of ACN were added. The mixture was vortexed and incubated for 10 min, then centrifuged for 15 min at 12 000 rpm. The supernatant was ready for derivatization.

2.7. Chromatography

The HPLC gradient system consisted of a Waters Model 6000 pump, a Waters Model 501 HPLC pump and a Waters Model 660 Solvent Programmer (Waters Corporation, Milford, MA, USA), a Rheodyne Model 7125 injector (Rheodyne Corporation, Cotati, CA, USA), a Spectra-Physics Model 100 UV detector (Thermo Separation Products, Fremont, CA, USA) and a Bioanalytical Systems Model LC-4B amperometric detector (Bioanalytical Systems, West Lafayette, IN, USA). A hydrodynamic cell, with a glassy carbon (GC) working electrode (Model MF-1000), a stainless steel counter electrode and a Ag/AgCl reference electrode was used. Data were

collected by a Linear recorder (Linear Instruments, Fremont, CA, USA) and a HP Model 3394A integrator (Hewlett Packard Corporation, Palo Alto, CA, USA). Phosphate buffer was made by titrating 1.0 *M* sodium phosphate (monobasic) solution to pH 3.0 with phosphoric acid. Mobile phase A consisted of the phosphate buffer–ACN–H_2O (2:2:96,v/v/v); mobile phase B consisted of the phosphate buffer–ACN–H_2O (2:60:38, v/v/v). All separations were generated on a YMC AP303, 300Å, 5μm ODS column (250×4.6 mm I.D.) (YMC, Wilmington, NC, USA) at room temperature and operated at a flow-rate of 1.5 ml/min. Gradient conditions: for AA derivatives, elution was performed initially at 100% mobile phase A for 2 or 5 min, then mobile phase B was linearly increased from 0% to 30% over 40 min and held for 10 min. For peptide elution, mobile phase B was linearly increased from 0% to 40% over 40 min. The UV detection wavelength was set at 254 nm and the applied potential for EC detection was set at +1.1 V, except for generating hydrodynamic voltammograms.

2.8. Cyclic voltammetry

The cyclic voltammetry system consisted of a Model CV-1B cyclic voltammograph, a voltammetry cell with a GC working electrode, a Ag/AgCl reference electrode, and a Pt auxiliary electrode (Bioanalytical Systems, West Lafayette, IN, USA). The sample was dissolved in the phosphate buffer which contained 20% ACN. The potential scans were performed between +1.5 and −1.0 V. The voltammograms were recorded by a Heathkit Model IR-5207 X-Y recorder.

2.9. Hydrodynamic voltammograms

Hydrodynamic voltammograms were constructed by obtaining multiple chromatograms of a given sample at 50 mV increments with the applied potential ranging from +0.85 V to +1.20 V. Signals were plotted vs. applied potential in the Figures.

3. Results and discussion

3.1. Cyclic voltammetry

The cyclic voltammogram (CV) for the authentic 6-AQC-Phe standard is shown in Fig. 1 (numbers 1–4 represent sequential scans). An oxidation wave was clearly seen at approximately +1.2 V. No reduction wave was observed. This result indicated that the oxidation of 6-AQC amine derivatives was chemically irreversible and involved a facile electron

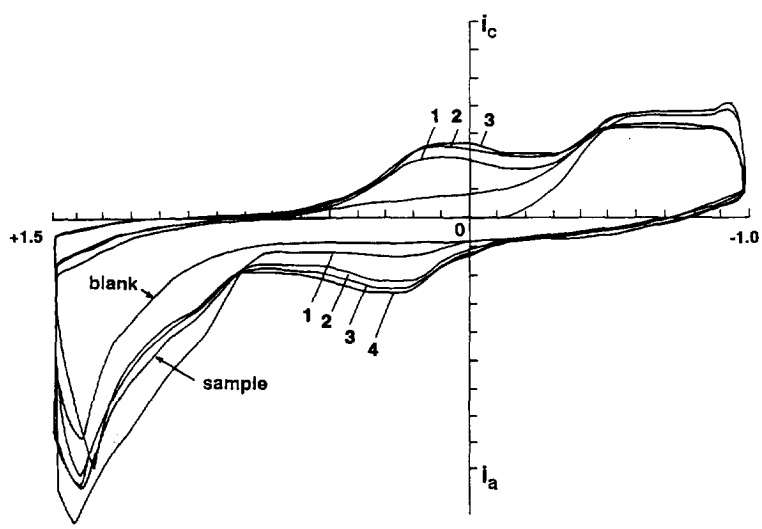


Fig. 1. Cyclic voltammograms of 6-AQC-Phe. The numbers 1, 2, 3 and 4 are the sequential scan numbers.

transfer. CVs of 6-AQC-Phe obtained from pH 2.3 to pH 7.0 were virtually identical, indicating that the oxidation mechanism involved no gain or loss of a proton. The repeat scans, however, yielded a new oxidation–reduction wave in the vicinity of +0.2 V in the CVs. This wave increased with the scan time, while the oxidation wave at +1.2 V decreased. This suggested that there was a chemical reaction following the EC oxidation and that the product of the reaction might be adsorbed on the surface of the GC working electrode. Mechanistic studies were not undertaken.

In an analogous study, Mathur et al. [35] have reported on the oxidation of 2-aminoquinoline at a stationary pyrolytic graphite electrode. While the current 6-AQC derivatives were not identical to 2-aminoquinoline, they appeared to follow very similar CV patterns. Over the entire pH range (2.2–10.4), 2-aminoquinoline was oxidized and exhibited a well-defined oxidation peak following a $2e^-$, $2H^+$ process that gave a dimer, 2,2′-azoquinoline, as the major product. The CV of 2-aminoquinoline, as a function of pH, showed an almost identical pattern of peaks and potentials to that obtained for 6-AQC-Phe (Fig. 1). The two peaks that appeared at lower working potentials have been shown to be interrelated (form a quasi-reversible couple). The product of the reaction corresponding to the peak at highest oxidative potential (+1.3 V) was electroactive and gave rise to one of the two peaks at lower potentials (0.0 V), at pH 6.8. At higher pH (10.4), these peaks were all shifted to the right by about −0.3 V. The influence of increasing concentrations of 2-aminoquinoline and of changing the scan rate, suggested strong adsorption of the initially formed species (+1.3V) at the surface of the electrode. All of these observations for 2-aminoquinoline and related amino heterocyclics parallel what we have observed for a typical 6-AQC-Phe derivative and the possible mechanisms operative in its CV electrochemistry.

3.2. Oxidation potential

Hydrodynamic voltammograms (HDVs) were constructed for nine 6-AQC derivatives of AAs. Most of the derivatives had similar half-wave potentials in the vicinity of +1.0 V (Fig. 2). However, some significant differences were apparent and the HDV for 6-AQC-Trp was different from the other derivatives. This extended voltammetric wave resulted from the presence of two electroactive moieties on 6-AQC-Trp, which differed slightly in oxidation potentials, although not enough to yield two distinct waves. 6-AQC-Tyr also contains two such sites, but did not show this effect, presumably because the oxidation potentials of the two sites were too similar. The fact that the response factors of Trp and Tyr derivatives were larger than those of other amino acid derivatives indicated that oxidation occurred at both electroactive sites.

HDVs of several 6-AQC derivatives of dipeptides were also constructed (Fig. 3). The half-wave potentials were still in the vicinity of +1.0 V, but the voltammograms were narrower than those from AAs. This suggested that perhaps the initial oxidation potentials may be increased with increasing peptide chain length.

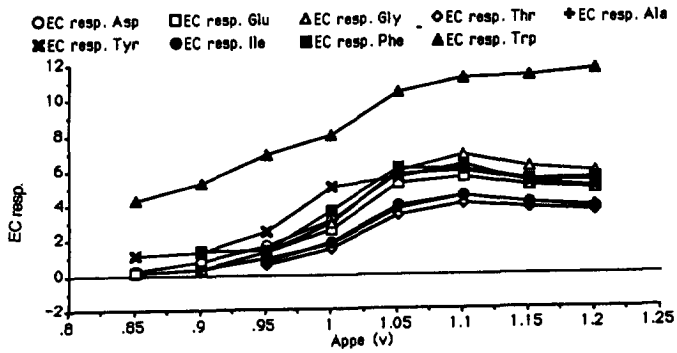


Fig. 2. Hydrodynamic voltammograms of 6-AQC derivatives of AAs.

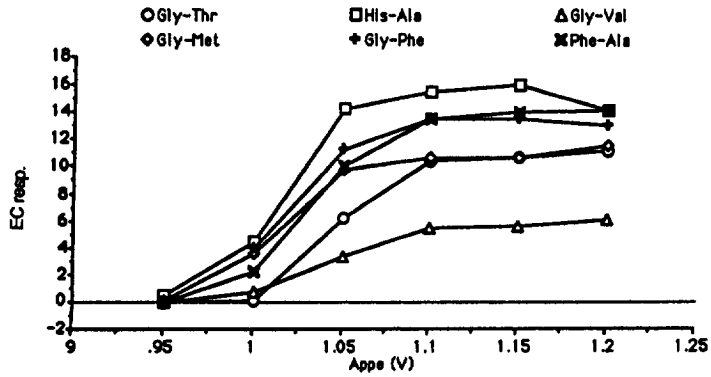


Fig. 3. Hydrodynamic voltammograms of 6-AQC derivatives of dipeptides.

3.3. Chromatography of derivatized AAs and dipeptides

The optimized separation of a derivatized standard mixture of 18 AAs was accomplished with gradient elution under acidic conditions (pH 3.0, Fig. 4). Operation at slightly lower pH values resulted in the co-elution of Arg with Gly. Operation at a higher pH, e.g., pH 5.0, similar to that previously reported, was not practical in these studies as it increased the retention of 6-AQ, the hydrolysis product of the reagent. This complicated the separation due to the large EC response of the reagent peak. The chromatogram of a derivatized standard dipeptide mixture is shown in Fig. 5. The gradient solvent conditions were stronger than those for AAs, in order to shorten the total elution time.

3.4. Detection limits and linear response of 6-AQC derivatives on an EC detector

Fig. 6 shows the chromatogram of a 2.5 pmol injection of a mixture of derivatized AAs. This was obtained by first derivatizing a higher concentration of the AA mixture (250 μM) and then performing serial dilutions to provide lower concentrations for analysis. As previously reported [19], a quantitative derivatization of each AA in the concentration range of 2.5–200 μM was assumed for these reactions. Based on this experiment, the EC detection limits of the derivatives could probably be lowered to a level of 1 pmol, while retaining a signal-to-noise ratio of 3:1. The detection limits might be lower if isocratic elution was used, because gradient elution made the baseline of the EC chromatogram noisier than usual.

Analysis of serial dilutions of a standard AA derivative mixture ranging from 5 pmol (0.5 μM) to

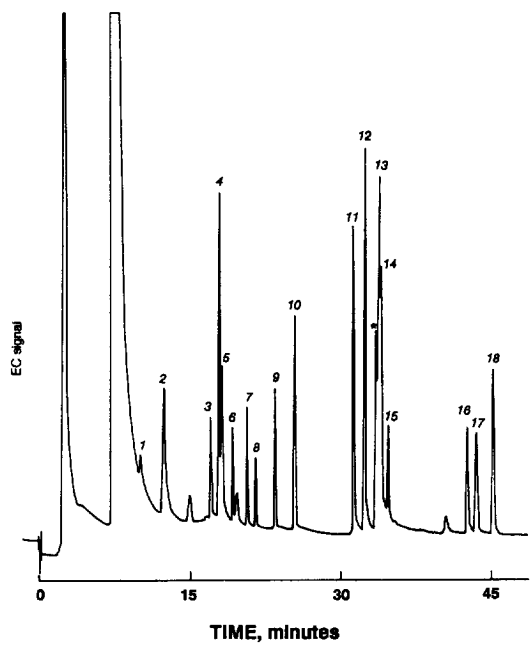


Fig. 4. Chromatogram of 6-AQC derivatives of AAs with EC detection. Applied potential: +1.10 V, GC working electrode, stainless steel auxiliary electrode and Ag/AgCl reference electrode. Gradient elution: 100% A was held for 2 min, then B was increased from 0% to 30% over 40 min and held for 10 min (see Section on chromatography Section 2.7). Flow-rate: 1.5 ml/min. Injection: 25 pmol each. 1, NH_3; 2, His; 3, Ser; 4, Arg; 5, Gly; 6, Asp; 7, Glu; 8, Thr; 9, Ala; 10, Pro; 11, Cys; 12, Lys; 13–14, Met and Tyr; 15, Val; 16, Ile; 17, Leu; 18, Phe.

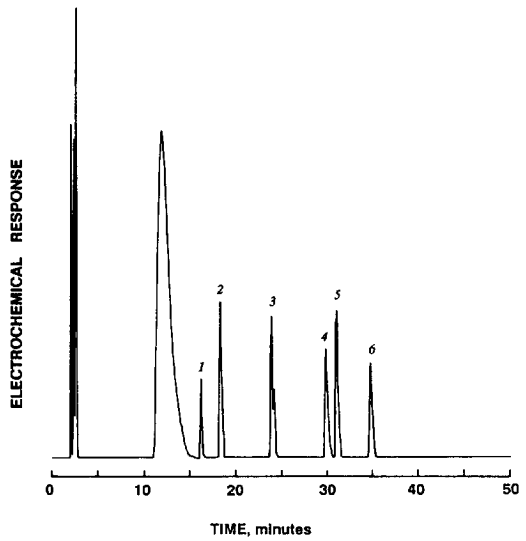


Fig. 5. Chromatogram of 6-AQC derivatives of dipeptides with EC detection. Gradient elution: mobile phase B was linearly increased from 0% to 40% over 40 min (See Section on chromatography Section 2.7). Flow-rate: 1.5 ml/min. Detection conditions are the same as in Fig. 4. 1, Gly-Thr; 2, His-Ala; 3, Gly-Val; 4, Gly-Met; 5, Gly-Phe; 6, Phe-Ala.

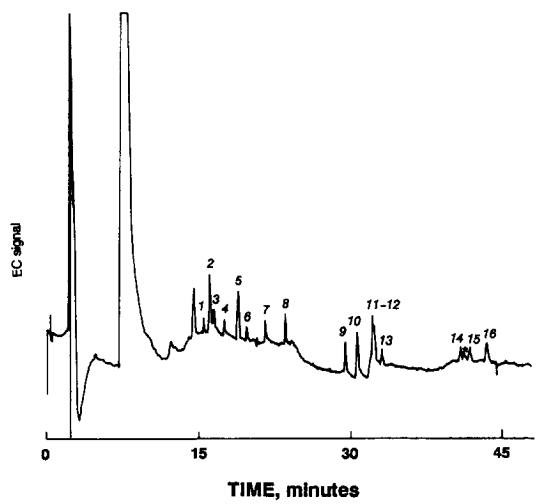


Fig. 6. Chromatogram of 6-AQC derivatives of AAs with EC detection. Applied potential: +1.10 V, GC working electrode, stainless steel auxiliary electrode and Ag/AgCl reference electrode. Gradient elution: 100% A was held for 5 min, then B was increased from 0% to 30% over 40 min and held for 10 min (See Section 2.7). Flow-rate: 1.5 ml/min. Injection: 2.5 pmol each. 1, Ser, 2; Arg, 3, Gly; 4, Asp; 5, Glu; 6, Thr; 7, Ala; 8, Pro; 9, Cys; 10, Lys; 11–12, Met and Tyr; 13, Val; 14, Ile; 15, Leu; 16, Phe.

Table 1
Linearity of 6-AQC AAs with EC detection[a,b]

Amino acid	a	b	r^2
Asp	0.985	5.18	0.991
Glu	1.009	5.07	0.997
Gly	1.002	5.19	0.999
Thr	1.094	4.637	0.995
Ala	0.998	5.065	0.996
Tyr	1.016	5.439	0.999
Ile	1.06	4.869	0.999
Phe	1.065	5.042	0.997
Trp	1.048	5.046	0.996

[a] Injection amount for each AA was from 5 pmol to 2 500 pmol. The linearity has been represented by an equation (EC signal=$a\times$ concentration of AA derivatives+b).

[b] Data obtained by derivatization at a high concentration of AAs followed by serial dilution and then LCEC analysis.

2 500 pmol (250 μM) yielded calibration curves with correlation coefficients (r^2)$\neq$ 0.99 (Table 1). This demonstrated that the linear response of the derivatives on an amperometric detector had a range of about three orders of magnitude. Others have already demonstrated that these same AAs, at the concentrations indicated in Table 1, are all quantitatively derivatized, even when such derivatizations are performed at different concentration levels [19]. Thus, our calibration studies were only designed to demonstrate EC linearity plots for the same concentration range of 6-AQ-AA derivatives.

3.5. Analysis of AAs in plasma

The EC detection of 6-AQC derivatives of AAs was applied to the determination of AAs in human and bovine plasma. Because the results obtained on both chromatograms were very similar, we provide only one, Fig. 7 (bovine plasma). The blank was clean and very few interferences were observed. Trp, difficult to detect with FL because of its self-quenching, was well-determined in this EC experiment. The quantitative results of some AAs in human and bovine plasma are shown in Table 2 and Table 3 (different samples). The data obtained from EC detection in the experiment shown in Table 3 agreed well with those presented in the literature [32,33]. There are some AAs that are significant in plasma, but were intentionally not identified or quantitated in these analyses, such as: Asn, Gln, Orn and Cit. It

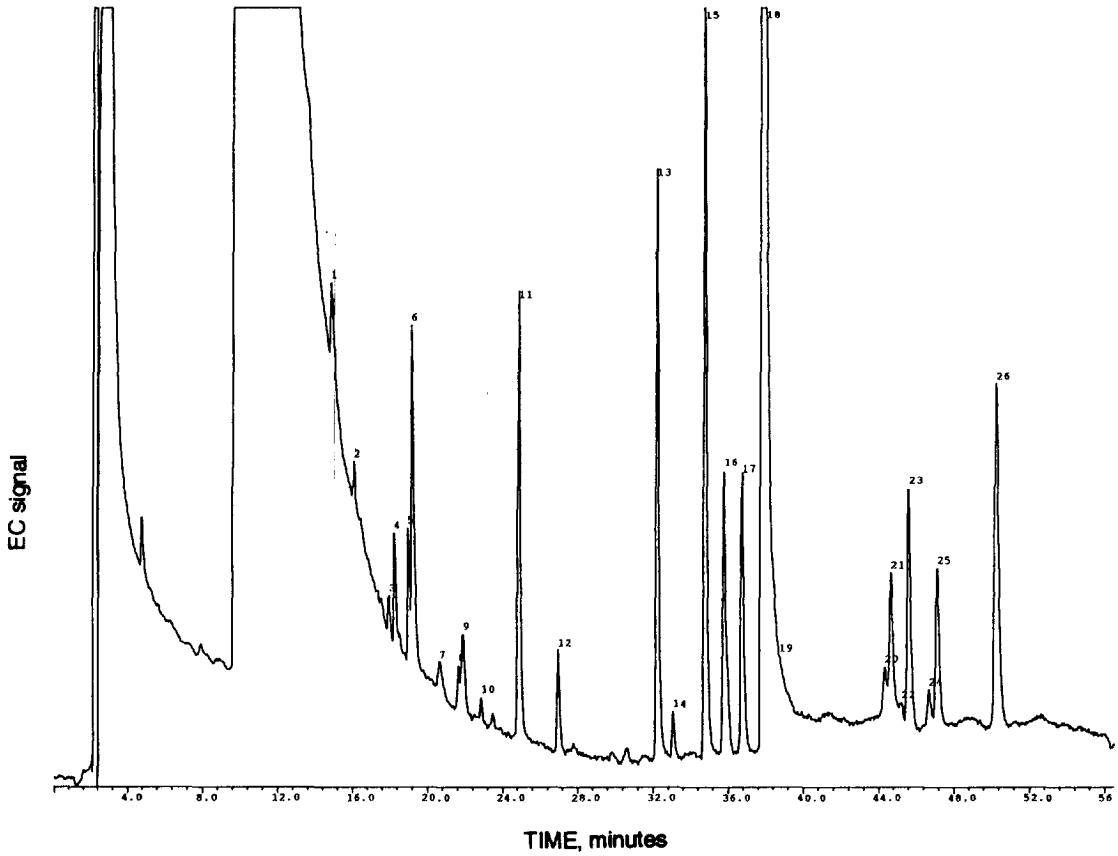


Fig. 7. Chromatogram of 6-AQC derivatives of AAs in bovine plasma. HPLC conditions are the same as in Fig. 4. 1, NH_3; 2, His; 4, Ser; 5, Arg; 6, Gly; 7, Asp; 9, Glu; 10, Thr; 11, Ala; 12, Pro; 14, Cys; 15, Lys; 17, Met + Tyr; 21, Ile; 23, Leu; 25, Phe; 26, Trp.

Table 2
Standard additions analysis of AAs in bovine plasma

Spiked (μM)	0	100	200	400
Found (μM)	x ± %R.S.D.[a] μM (mg/100ml)	x ± %R.S.D.[a,b]	x ± %R.S.D.[a,b]	x ± %R.S.D.[a,b]
Ala	340±6.2 (3.02)	111±2.7	197±3.1	378±2.2
Pro	54±4.8 (0.62)	109±5.0	190±7.2	357±7.3
Val	144±4.9 (1.68)	88±5.1	171±10.7	320±6.5
Ile	30±5.7 (0.39)	83±9.7	140±7.6	406±6.2
Leu	182±9.6 (2.38)	72±6.1	136±6.4	320±7.3
Phe	44±2.5 (0.73)	103±4.9	193±6.1	367±9.1
Trp	14±7.9 (0.29)	100±5.7	207±9.2	402±2.2

[a] x is the average number, %R.S.D. is percent relative standard deviation, $n=3$.
[b] Values were derived by subtracting the amount found in the unspiked sample from each spiked sample.
[c] Sample assayed here was different from that in Table 3.

Table 3
Concentration of AAs in plasma[a]

Amino acid	Bovine	Human	Literature [33] (human)
Ser	1.16	2.50	0.77–1.76
Arg	0.78	0.85	0.37–2.40
Gly	1.26	1.17	0.90–4.16
Ala	1.93	1.80	1.87–5.89
Pro	0.57	1.20	1.17–3.87
Lys	1.43	1.49	1.21–3.48
Ile	0.93	0.66	0.48–1.28
Leu	1.64	1.07	0.98–2.30
Phe	0.88	0.52	0.61–1.45

[a] Concentration is represented as mg/100ml.
[b] Sample assayed here was different from that in Table 2.

was not our intention to demonstrate quantitation for all possible AAs in plasma, but only to demonstrate the feasibility of determining typical ones, as desired. In Table 2, there are two large discrepancies (spiked levels, standard additions, 100–200 μM) for Leu and Ile. This may be the result of sample preparation (recovery from more dilute solutions may not be quantitative), or other reasons, as yet unknown. Nevertheless, the final levels incurred are still within the normal range expected for Leu and Ile (Table 3), probably because of the very nature of the standard additions method of quantitation.

3.6. Compositional analysis of proteins

AA compositional data for hydrolyzed BSA are shown in Table 4 and Fig. 8. There is excellent agreement for most AAs with the compositions published in the literature [32]. The reproducibility and accuracy of the EC detection for all AAs are at the same levels as those found by UV. Some small errors between the experimental results and the literature values (e.g., Ser) may derive from sample preparation. Though not shown, it is possible to use the same 6-AQC derivatization and LCEC approaches for a trypsin-TPCK digested cyt *c*. Although the peaks in the generated chromatogram were not identified, the qualitative results again demonstrated that the EC method could be used for the detection of small peptide, 6-AQC derivatives [30,31].

4. Conclusions

6-AQC derivatives of AAs and peptides showed EC activity because the compounds contained an aromatic amine (aminoquinoline) structure. This property could be applied to HPLC with EC detection. The response of the derivatives on an

Table 4
Compositional analysis of hydrolyzed BSA[a]

Amino acid	Actual	Number (UV)[c]	%R.S.D.[d]	Number (EC)[c]	%R.S.D.[d]
His	17	15.1	4.8	16.1	4.8
Ser[b]	28	21.4	2.2	20.4	3.3
Arg	23	23.9	5.0	22.3	2.4
Gly	16	17.6	3.3	17.0	1.5
Asp	54	57.8	3.1	48.8	2.0
Glu	79	76.3	2.0	67.5	1.0
Thr	34	30.5	0.8	31.7	1.7
Ala	46	48.3	1.2	47.5	1.6
Pro	28	29.5	1.7	30.0	1.1
Lys	59	60.9	2.3	61.3	1.2
Val	36	35.4	1.2	36.0	2.6
Ile	14	14.0	1.9	14.6	3.5
Leu	61	62.1	1.3	65.0	3.1
Phe	27	26.4	1.1	28.8	3.3

[a] Values are given as residues per mol of protein.
[b] Ser is known to be partially destroyed by hydrolysis, which would account for the lower value determined here [32].
[c] Data obtained in this study, from the very same sample injection, simultaneous UV/EC detection occurred for each injection.
[d] Values are given as average (n=3) and percent relative standard deviation (%RSD).

amperometric detector was studied in the range of 5 pmol to 2,500 pmol, and the detection limits were at about the 2.5 pmol level. There is generally good agreement in the experimental results with literature values for the quantitative levels of AAs in plasma and in a BSA-hydrolysate. These overall results have now demonstrated the practical utility of detecting 6-AQC derivatives electrochemically in HPLC and most likely in HPCE.

OPA-AAs can be detected in LCEC at a somewhat lower potential, perhaps +0.7–0.8 V, making that approach, at present, a bit more selective. It is also possible that, regardless of the mobile phase or buffer, these 6-AQ AAs will always require a higher oxidative potential for their detection than the analogous OPA ones. Further work would be required to answer this point. It is clear that these 6-AQ derivatives presently require a high working potential, which is a disadvantage when compared with the analogous OPA or NDA derivatives. Also, the presence of a significant interfering peak due to unreacted 6-AQC or its hydrolysis products, such as 6-AQ itself, which are highly EC active, present potentially serious disadvantages in using this reagent for trace assays, as resolutions are incomplete or not fully baseline from the hydrolysis products. Because OPA and/or NDA reagents provide derivatives that can be detected at a lower working potential and therefore more selectively, and because of the absence of EC detector properties for unreacted reagent, the 6-AQC approach is less than ideal at the present time. The current studies provide and alternative EC detection method for applications that currently use 6-AQC for UV–FL detection. It has also been shown that most 6-AQ urea derivatives are far more stable than the OPA or NDA analogous derivatives, especially when formed precolumn. This is an advantage for the current 6-AQC approach in LCEC, despite the above negative aspects.

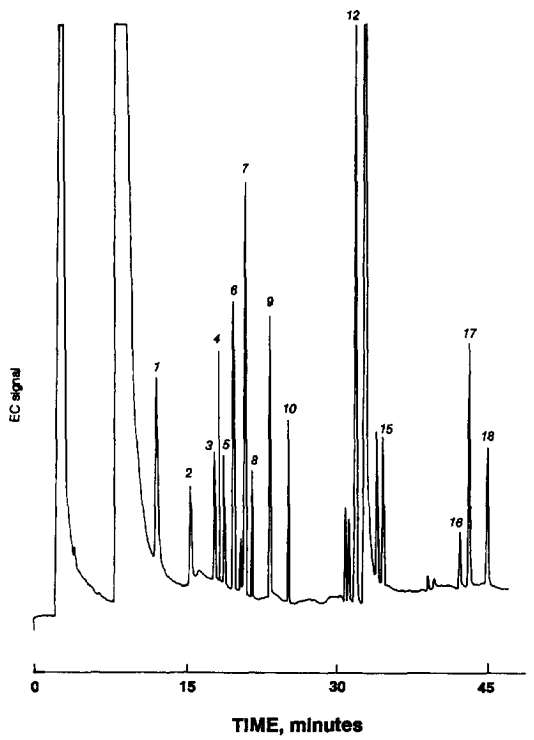


Fig. 8. Chromatogram of 6-AQC derivatized BSA hydrolysate. HPLC conditions are the same as in Fig. 4. 1, NH_3; 2, His; 3, Ser; 4, Arg; 5, Gly; 6, Asp; 7, Glu; 8, Thr; 9, Ala; 10, Pro; 12, Lys; 15, Val; 16, Ile; 17, Leu; 18, Phe.

5. Glossary

AA(s)	amino acid(s)
ACN	acetonitrile
AcOH	acetic acid
6-AQC	6-aminoquinolyl-N-hydroxy-succinimidyl carbamate
6-AQ	6-aminoquinoline
BSA	bovine serum albumin
cyt *c*	cytochrome *c* (horse heart)
CV	cyclic voltammogram or voltammetry
Dansyl-Cl	1-dimethylaminonaphthalene-5-sulfonyl chloride
Dabsyl-Cl	4-dimethylaminoazobenzenesulfonyl chloride
DMF	dimethyl formamide
EC	electrochemistry or electrochemical detection
FL	fluorescence detection
9-FMOC	9-fluorenylmethyl chloroformate
GC	glassy carbon working electrode material
HPLC	high performance liquid chromatography
HDV	hydrodynamic voltammogram

H_2O	water
LCEC	liquid chromatography with electrochemical detection
NDA	naphthalene-2,3-dicarboxaldehyde
NBD-F	7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole
OPA	*ortho*-phthalaldehyde
Phe	L-phenylalanine
PITC	phenylisothiocyanate
(%)R.S.D.	(percent) relative standard deviation
rt	room temperature
$SOCl_2$	thionyl chloride
TEA	triethylamine
trypsin-TPCK	trypsin-L-1-tosylamido-2-phenylethyl chloromethyl ketone

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