Assay of advanced glycation endproducts (AGEs): surveying AGEs by chromatographic assay with derivatization by 6-aminoquinolyl-*N*-hydroxysuccinimidyl-carbamate and application to N_ε-carboxymethyl-lysine- and N_ε-(1-carboxyethyl)lysine-modified albumin

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Glycation of proteins leads to the formation of early glycation adducts (fructosamine derivatives) and advanced glycation endproducts (AGEs). Formation of AGEs has been linked to the development of cataract, diabetic complications, uraemia, Alzheimer's disease and other disorders. AGEs are a group of compounds of diverse molecular structure and biological function. To characterize AGE-modified proteins used in studies of structural and functional effects of glycation, an assay was developed that surveys the content of early and advanced glycation adducts in proteins. The assay procedure involved enzymic hydrolysis of protein substrate, derivatization of the hydrolysate with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) and HPLC of the resulting adducts with fluorimetric detection. Structural isomers of methylglyoxal-derived hydroimidazolone, glyoxal-derived hydroimidazolone, 3-deoxyglucosone-derived hydroimidazolone and N_δ-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidin-2-yl)-ornithine (THP) were determined for the first time. AGEs with intrinsic fluorescence (argpyrimidine, pentosidine) were assayed without derivatization. Limits of detection were 2–17 pmol and levels of recovery were 50–99 %, depending on the analyte. The AQC assay resolved structural and epimeric isomers of methylglyoxal-derived hydroimidazolones and THP. Hydroimidazolones, THP and argpyrimidine were AGEs of short-to-intermediate stability under physiological conditions, with half-lives of 1–2 weeks. Their measurement provides further insight into the glycation process. The assay was applied to the characterization of human serum albumin minimally and highly modified by N_ϵ-carboxymethyl-lysine and N_ϵ-(1-carboxyethyl)-lysine.

Key words: 3-deoxyglucosone, fructosamine, glucose, glyoxal, methylglyoxal.

INTRODUCTION

Glycation of proteins is the non-enzymic reaction of glucose, α-oxoaldehydes and other saccharide derivatives with proteins. It involves a complex series of parallel and sequential reactions, often termed collectively 'the Maillard reaction'. This forms many different adducts, some of which are fluorescent and coloured [1,2]. Historically, glycation of lysyl side chain and N-terminal amino groups by glucose was the major glycation process studied. Glucose reacts with amino groups to form initially a Schiff's base, which undergoes an Amadori rearrangement to form *N*-(1-deoxy-D-fructos-1-yl)-amino acid or fructosamine [3] (Figure 1a). Reactive α-oxoaldehydes (glyoxal, methylglyoxal and 3-deoxyglucosone) are also important precursors of advanced glycation endproduct (AGEs) *in vivo* [2,4–7] (Figure 1b). Early glycation adducts are fructosamine derivatives of N-terminal amino groups and lysyl side chains of proteins (Figure 1c). AGEs are other glycation adducts [1] (Figure 1d). 'Glycoxidation' is a term used for glycation processes involving oxidation. The AGEs thereby formed are called 'glycoxidation products' [8]. Pentosidine and N_ϵ-carboxymethyl-lysine (CML) are glycoxidation products. There are at least four processes involved in the formation of AGEs in physiological systems: (i) monosaccharide autoxidation (autoxidative glycosylation), or the degradation of saccharide unattached to a protein [9–12], (ii) Schiff's base fragmentation [10,13], (iii) fructosamine degradation [12,14,15] and (iv) direct reaction of α,β-dicarbonyl compounds formed from the degradation of glycolytic intermediates and lipid peroxidation with proteins [7,16,17] (Figure 1a).

The extent of glycation of proteins in physiological systems is typically 0.01–1 % of lysine and arginine residues. For human serum albumin (HSA) in blood plasma, the concentration ranges (mol/mol of albumin) of Schiff's base adduct, fructosamine and AGEs are $\approx$ 1–5, 6–15 and 0.01–7 %, respectively [10,18,19]. Higher extents of glycation have been produced in proteins glycated *in vitro* where almost exhaustive glycation of lysine and arginine residues has been achieved in some instances [20,21].

Many AGEs have been found in physiological systems: hydroimidazolones derived from methylglyoxal, glyoxal and 3-deoxyglucosone [16,22–25], CML [15], N_ϵ-(1-carboxyethyl)-lysine (CEL) [26], pyrraline [27], the bis(lysyl) imidazolium derivatives MOLD [methylglyoxal-derived lysine dimer, 1,3-di(N^ϵ-lysino)-4-methyl-imidazolium salt], GOLD [glyoxal-derived lysine dimer, 1,3-di(N^ϵ-lysino)imidazolium salt] and DOLD [3-deoxygluco-

Abbreviations used: AGE, advanced glycation endproduct; AQC, 6-aminoquinolyl-*N*-hydroxysuccinimidyl-carbamate; CEL, N_ϵ-(1-carboxyethyl)lysine; CML, N_ϵ-carboxymethyl-lysine; DETAPAC, diethylenetriaminepenta-acetic acid; 3DG-H1, N_δ-[5-(2,3,4-trihydroxybutyl)-5-hydro-4-imidazolon-2-yl] ornithine; DOLD, 3-deoxyglucosone-derived lysine dimer, 1,3-di(N^ϵ-lysino)-4-(2,3,4-trihydroxybutyl)-imidazolium salt; G-H1, N_δ-(5-hydro-4-imidazolon-2-yl)ornithine; GOLD, glyoxal-derived lysine dimer, 1,3-di(N^ϵ-lysino)imidazolium salt; HSA, human serum albumin; IS, internal standard; LOD, limit of detection; MALDI-MS, matrix-assisted laser-desorption ionization MS; MG-H1, N_δ-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine; MG-H2, 2-amino-5-(2-amino-5-hydro-5-methyl-4-imidazolon-1-yl)pentanoic acid; MG-H3, 2-amino-5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl)pentanoic acid; MOLD, methylglyoxal-derived lysine dimer, 1,3-di(N^ϵ-lysino)-4-methyl-imidazolium salt; TFA, trifluoroacetic acid; THP, N_δ-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidin-2-yl)-ornithine; t-Boc, t-butyloxycarbonyl.

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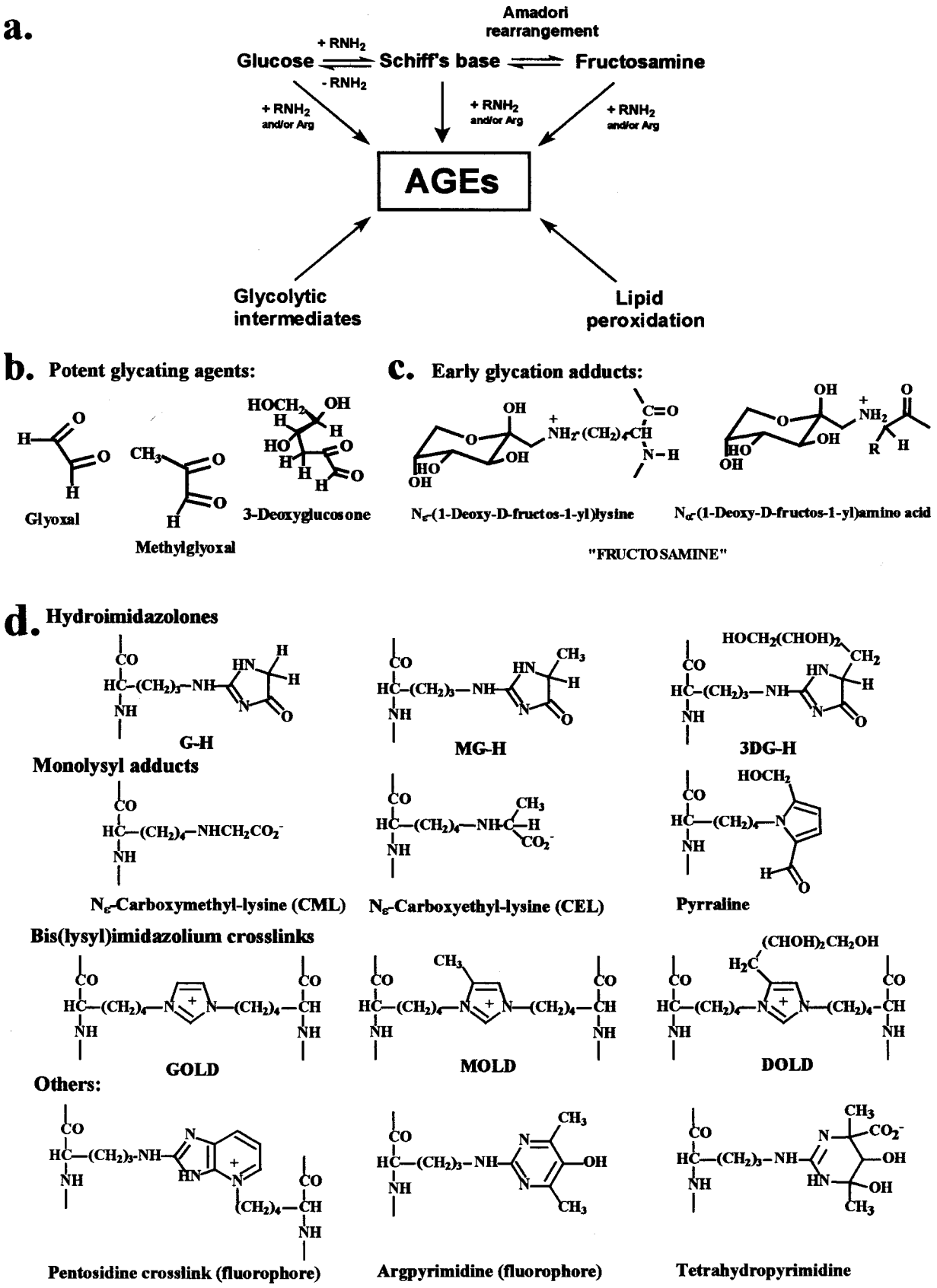

Figure 1 Formation of early glycation adducts and AGEs

(**a**) Formation of AGEs. (**b**) Reactive α-oxoaldehydes in physiological glycation processes. Unhydrated, acyclic forms are shown but hydrates, dimers and cyclic forms are the major species in solution. (**c**) Early glycation adducts (fructosamines). (**d**) Molecular structures of AGEs. Only isomers 1 of hydroimidazolone AGEs are shown.

sone-derived lysine dimer, 1,3-di(N^{ε}-lysino)-4-(2,3,4-trihydroxybutyl)-imidazolium salt] [28,29], pentosidine [30], argpyrimidine [31] and arginine-derived N_{δ}-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidin-2-yl)-ornithine (tetrahydropyrimidine, THP) [16] (Figure 1d). AGEs have different biological and physiological functions: some are protein cross-links (pentosidine, MOLD, GOLD and DOLD), some are recognition factors for specific AGE-binding cell-surface receptors (CML, methylglyoxal-derived hydroimidazolone) and some are markers and risk predictors of disease processes [32–34]. Measurement of these different AGEs discretely in proteins glycated *in vitro* and *in vivo* is important to enable a correlation of AGE composition and content of proteins with biochemical and physiological function.

Glycation adducts are assayed by chromatographic and immunochemical techniques [15,25,26,28,35–38]. In this report, we describe the preparation of AGE analytical standards and their application for the analysis of fructosyl-lysine and AGEs in enzymic hydrolysates of proteins by the 6-aminoquinolyl-*N*-hydroxysuccinimidyl-carbamate (AQC)-derivatization technique [39]. AGEs with intrinsic fluorescence were also assayed chromatographically without derivatization. The application of the technique to the study of AGEs in HSA prepared with minimal and high contents of CML and CEL is described herein and application to proteins glycated minimally and highly by methylglyoxal and glucose *in vitro* is described in the accompanying report [40].

MATERIALS AND METHODS

Materials

L-Amino acids (SigmaUltra; > 99 % purity), N_{α}-t-Boc-ornithine (where t-Boc is t-butyloxycarbonyl), N_{α}-t-Boc-arginine and N_{α}-t-Boc-lysine were purchased from Sigma. Ammonium isothiocyanate, acetic anhydride, pyruvate methyl ester and $NaBH_3CN$ were purchased from Aldrich. Columns for reversed-phase HPLC were: analytical scale, 3.9 mm × 150 mm, NOVAPAK™ ODS, 4 μm, fitted with a 3.9 mm × 20 mm NOVAPAK™ ODS Sentry guard column at 34 °C and a flow rate of 1 ml/min; and preparative scale, 2.5 mm × 10 cm NOVAPAK™ ODS with 2.5 cm × 1 cm guard column cartridge in a 25 × 10 radial compression unit (Waters-Millipore) at room temperature with a flow rate of 9.9 ml/min. ^{1}H and ^{13}C NMR (proton decoupled) spectra were recorded on a Jeol EX 270 MHz spectrometer. Low-resolution matrix-assisted laser-desorption ionization MS (MALDI-MS) was performed with a Kratos Kompact MALDI II mass spectrometer equipped with a nitrogen laser (337 nm), operating in positive high-energy linear mode. The matrix solution contained 10 mg/ml 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) in 60 % (v/v) acetonitrile in 0.1 % (v/v) aqueous trifluoroacetic acid (TFA). Analytical TLC was performed using silica gel F_{254} plates (Merck). Methylglyoxal and AQC were prepared as described previously [41].

Preparation of analytical standards for the chromatographic assay of AGEs

Methylglyoxal-derived hydroimidazolones: N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1; isomer 1; Figure 2a)

L-Alanine (1.78 g, 20 mmol) and ammonium isothiocyanate (1.52 g, 20 mmol) were dissolved in acetic anhydride (10 ml) and acetic acid (1 ml) and heated at 100 °C for 45 min. The product mixture was cooled and poured on to iced water (50 ml). The precipitate formed was collected by filtration and recrystallized from ethanol. The product, 3-acetyl-5-methyl-2-thioxo-4-imidazolidinone, was characterized by ^{1}H NMR ([^{2}H]methanol) where chemical shift δ_H (p.p.m.) values were: 2.7 (s, 3-acetyl, 3H), imidazolidinone ring 1.42 (d, 3H, 5-CH_3) and 4.67 (q, 5-H) and 12.6 (s, -NH). The melting point was 165 °C, TLC (silica gel; mobile phase, chloroform/ethanol/methanol, 8:1:1, by vol.) gave $R_f = 0.7$, and the yield was 0.58 g (17 %).

3-Acetyl-5-methyl-2-thioxo-4-imidazolidinone (1.93 g, 11.2 mmol) was then suspended in 10 % aqueous HCl (12 ml) and heated to 100 °C for 45 min. The resulting solution was cooled, and the product was crystallized, isolated by filtration, washed with water and dried. The product, 5-methyl-2-thioxo-4-imidazolidinone, gave ^{1}H NMR ([^{2}H]methanol) δ_H values of 1.23 (d, 3H, 5-CH_3), 4.24 (q, 5-H), 10.01 (s, -NH) and 11.64 (s, -NH). The melting point was 160 °C and the yield was 1.09 g (75 %).

5-Methyl-2-thioxo-4-imidazolidinone (0.33 g, 2.5 mmol) and anhydrous potassium carbonate (0.36 g, 1.5 mmol) were suspended in acetonitrile (25 ml) and iodomethane (0.45 g, 3 mmol) added. The mixture was stirred for 4 h at room temperature and then the precipitate removed by filtration. The filtrate was evaporated to dryness *in vacuo* and the residual solid crystallized from methanol to give the product, 5-hydro-5-methyl-2-methylthio-4-imidazolone. ^{1}H NMR (CD_3CN) gave the following δ_H values: hydroimidazolonyl 1.33 (d, 3H, 5-CH_3), and 3.99 (q, 5-H), 2.51 (s, 3H, -SCH_3) and 12.6 (s, -NH). The melting point was 157 °C (associated with decomposition), TLC analysis (silica gel; chloroform/ethanol, 9:1, v/v) gave an R_f value of 0.58, and the yield was 310 mg (86 %).

Finally, 5-hydro-5-methyl-2-methylthio-4-imidazolone (154 mg, 1.0 mmol) and N_{α}-t-Boc-ornithine (232 mg, 1.0 mmol) were dissolved in methanol (10 ml) and sodium methoxide (1.0 M, 1.0 ml) added. The solution was refluxed for 6 h, cooled and the solvent evaporated *in vacuo*. The solid residue was washed four times with 4 ml of acetonitrile and dried to give the crude product (300 mg). This was purified by preparative reversed-phase HPLC. The flow rate was 9.9 ml/min. The mobile phase was 0.1 % TFA in water with a linear gradient of 0–80 % methanol from 0 to 40 min. The eluate was monitored by absorbance at 210 nm. Eluate corresponding to the peak absorbing in the retention time interval 20–21 min was collected and lyophilized to dryness to give a white solid product (13 mg). This was stirred with ice-cold TFA (30 μl) for 15 min and then evaporated to dryness *in vacuo* to give the product (9.12 mg; yield, 4 %).

MG-H1 had the following characteristics. ^{1}H NMR ([^{2}H]methanol) analysis gave the following δ_H (p.p.m.) and coupling constant J (Hz) values: imidazolonyl 1.43, $J = 6.9$ (d, 3H, 5-CH_3) and 4.41, $J = 6.9$ (q, 1H, 5-H); ornithyl 1.8–2.0 (m, 4H, 3-H and 4-H), 3.37 (t, 2H, 5-H) and 3.97, $J = 6.3$ (t, 1H, 2-H). ^{13}C NMR chemical-shift δ_C (p.p.m.) values were: imidazolonyl 157.1 C-2, 178.3 C-4, 56.8 C-5 and 16.6 5-CH_3; ornithyl 173.0 C-1, 53.8 C-2, 28.2 C-3, 24.8 C-4 and 42.7 C-5. MALDI-MS analysis gave a molecular ion $(M+1)$ with $m/z = 229$. Analytical reversed-phase HPLC analysis (mobile phase, 10 mM sodium phosphate buffer, pH 7.4; flow rate, 1 ml/min; absorbance detection at 210 nm) indicated the presence of only one compound, MG-H1, at a retention time of 1.9 min.

Preparation of methylglyoxal-derived hydroimidazolones: 2-amino-5-(2-amino-5-hydro-5-methyl-4-imidazolon-1-yl)pentanoic acid (MG-H2; isomer 2; Figure 2b)

N_{α}-t-Boc-ornithine (232 mg, 1.0 mmol), methylpyruvate (200 μl, 2.2 mmol) and $NaBH_3CN$ (140 mg, 2.2 mmol) were dissolved in methanol (9 ml) and stirred at room temperature for 3 days. The solvent was then evaporated *in vacuo*. The solid residue was

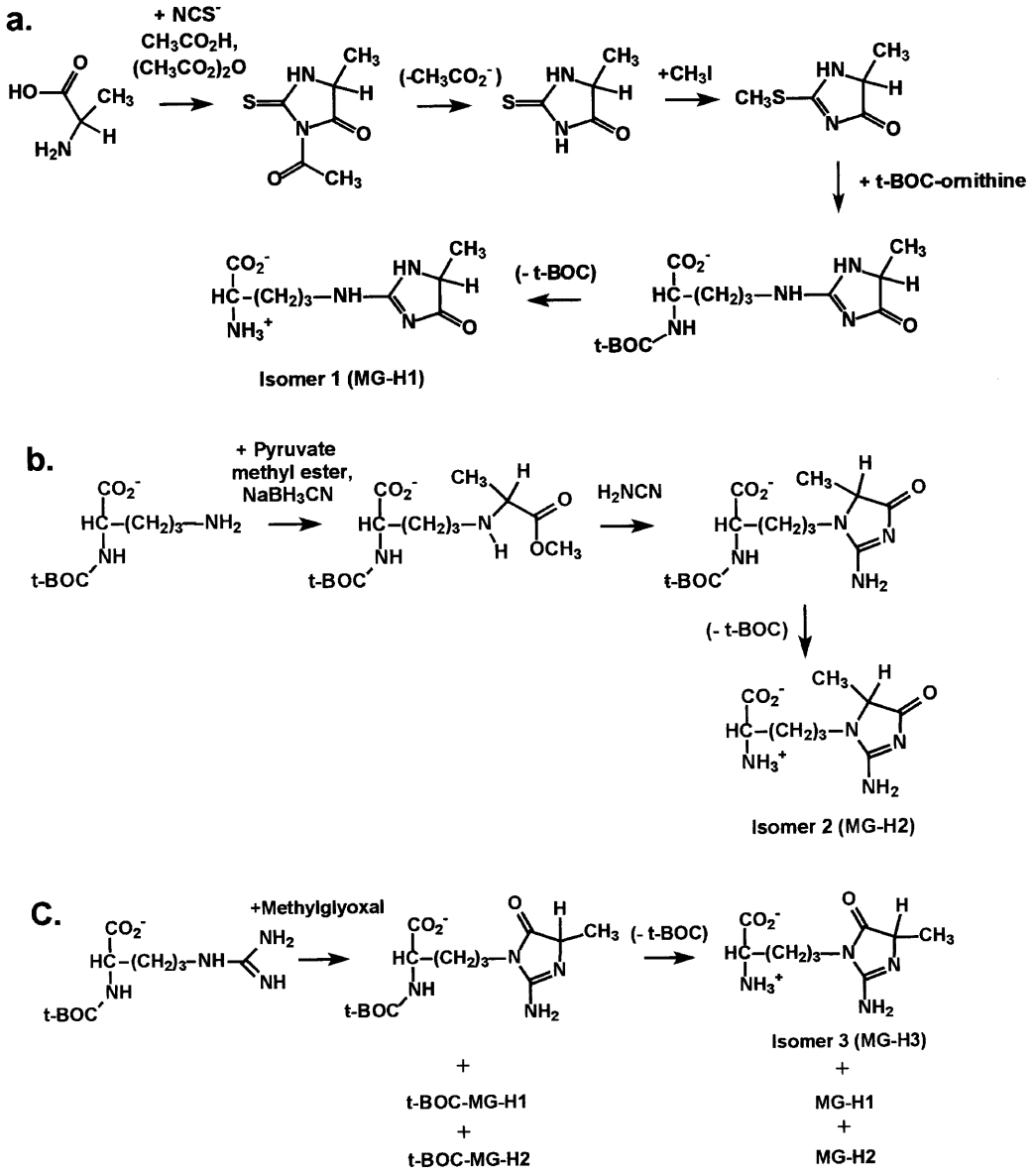

Figure 2 Preparation of methylglyoxal-derived hydroimidazolones

(**a**) Isomer 1, MG-H1; (**b**) isomer 2, MG-H2; (**c**) isolation of isomer 3, MG-H3.

dissolved in water (4 ml) and acidified with HCl (200 mM) to pH 3. This was stirred at room temperature for 5 h to remove $NaBH_3CN$ and then lyophilized to dryness. The solid residue was extracted with ethanol (six times, 2 ml) and the solvent removed from the combined extracts *in vacuo* to give the crude product (226 mg). This was purified by preparative reversed-phase HPLC. The mobile phase was 0.1 % TFA in water with a linear gradient of 0–100 % methanol over 0–50 min. The flow rate was 9.9 ml/min. The sample loading was 100 mg of crude solid in 0.1 % TFA in water (1.0 ml) and the eluate was monitored by absorbance at 225 nm. Eluate corresponding to the peak absorbing in the retention time interval 28–35 min was collected and lyophilized to dryness to give the product, N_α-t-Boc-N_δ-(methylpropion-2-yl)ornithine. ^{1}H NMR ([^{2}H]methanol) analysis gave δ_H values of: 1.44 (s, 9H, t-Boc), 1.60 (d, 3H, -CH_3), 4.12 (q, -H) and 3.84 (s, 3H, -OCH_3); ornithyl 1.70–1.92 (m, 4H, 3-CH_2 and 4-CH_2), 3.30 (t, 2H, 5-H), 3.78 (t, 2-H) and 3.01 (br., 5-NH). MALDI-MS analysis gave a molecular ion ($M+1$) with an m/z value of 317. The yield was 54 mg (14 %).

N_α-t-Boc-N_δ-(methylpropion-2-yl)ornithine (54 mg, 0.14 mmol) was dissolved in 0.5 M sodium methoxide in methanol (2 ml), cyanamide (50 mg, 1.2 mmol) added and stirred for 4 days at room temperature. The solvent was evaporated *in vacuo* to give the crude product (50 mg) that was purified by reversed-phase HPLC. The mobile phase was isocratic 0.1 % TFA in water, the flow rate was 9.9 ml/min and the sample loading was 50 mg in 0.5 ml of mobile phase. The eluate was monitored by absorbance at 210 nm, and fraction corresponding to the absorbance peak in the retention interval 13–17 min collected and lyophilized to dryness. The solid residue was stirred with ice-cold TFA (62 μl) for 15 min and the TFA evaporated *in vacuo* to give 26 mg of the product (yield, 68 %). MG-H2 had the

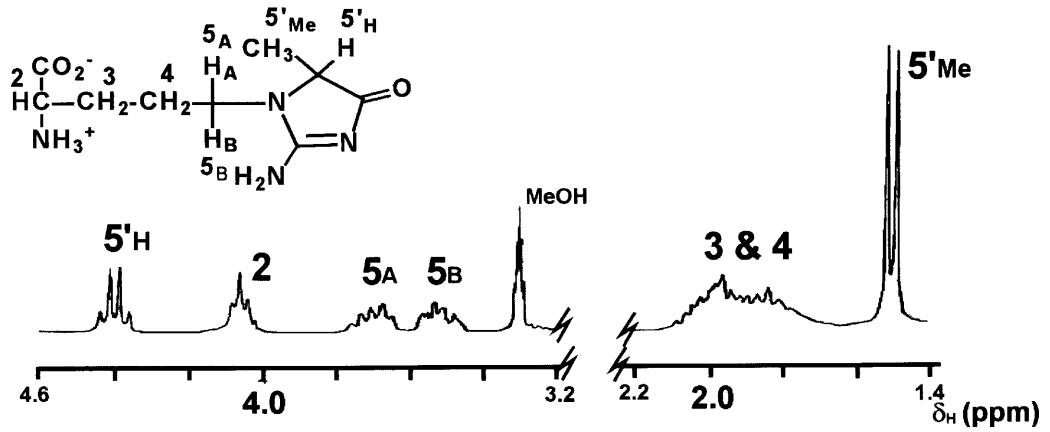

Figure 3 ^{1}H NMR spectrum of methylglyoxal-derived hydroimidazolone isomer 2 (MG-H2)

^{1}H NMR spectra were recorded in [^{2}H]methanol. The chemical-shift standard was tetramethylsilane.

following characteristics. ^{1}H NMR (270 MHz, [^{2}H]methanol): imidazolonyl 1.52, $J = 7.3$ (d, 3H, 5-CH_3) and 4.40, $J = 7.3$ (q, 1H, 5-H); ornithyl 1.8–2.0 (m, 4H, 3-H and 4-H), 3.53 (m, 1H, 5-H_A), 3.69 (m, 1H, 5-H_B) and 4.07, $J = 6.0$ (t, 1H, 2-H) (Figure 3). ^{13}C NMR (68 MHz, [^{2}H]methanol, proton decoupled) chemical-shift δ_C (p.p.m.) values: imidazolonyl 158.8 C-2, 178.7 C-4, 61.8 C-5 and 16.5 5-CH_3; ornithyl 174.4 C-1, 55.3 C-2, 29.5 C-3, 25.3 C-4 and 44.6 C-5. MALDI-MS analysis gave a molecular ion ($M+1$) with $m/z = 229$. Analytical reversed-phase HPLC analysis (see conditions for MG-H1, above) indicated the presence of only one compound, MG-H2, at a retention time of 1.9 min.

Preparation of methylglyoxal-derived hydroimidazolones: 2-amino-5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl)pentanoic acid (MG-H3; isomer 3; Figure 2c)

N_α-t-Boc-arginine (0.5 mmol, 155 mg) and methylglyoxal (0.6 mmol) were dissolved in 30 ml of 0.2 M sodium acetate buffer, pH 5.4 and 37 °C, and incubated for 7 days under aseptic conditions. The product mixture was then lyophilized to dryness and the solid residue extracted with methanol (once with 30 ml, twice with 10 ml). Methanol was removed from the combined extracts *in vacuo* to give the crude product (196 mg). This was purified by preparative reversed-phase HPLC. The mobile phase was 0.1 % TFA in water with 20 % methanol (0–10 min) with a linear gradient of 20–50 % methanol (10–25 min). The flow rate was 9.9 ml/min. The sample loading was 50 mg in 0.5 ml of 0.1 % TFA in water. The eluate absorbance was monitored at 225 nm. Eluate corresponding to the absorbance peak in the retention interval 18–21 min was collected and lyophilized to dryness. The solid residue was dissolved in 0.5 M HCl (0.25 ml), stirred overnight at room temperature and lyophilized to dryness to yield the product (40 mg; yield, 35 %). MG-H3 had the following characteristics. ^{1}H NMR (270 MHz, [^{2}H]methanol): imidazolonyl 1.47, $J = 6.9$ (d, 3H, 5-CH_3) and 4.40, $J = 6.9$ (q, 1H, 5-H); ornithyl 1.8–2.0 (m, 4H, 3-H and 4-H), 3.76 (m, 2H, 5-CH_2) and 4.04, $J = 6.0$ (t, 1H, 2-H) (Figure 4, bottom panel). ^{13}C NMR (68 MHz, [^{2}H]methanol, proton decoupled): imidazolonyl 159.5 C-2, 175.6 C-4, 56.5 C-5 and 16.6 5-CH_3; ornithyl 171.4 C-1, 55.6 C-2, 28.5 C-3, 24.8 C-4 and 40.2 C-5. MALDI-MS analysis gave a molecular ion ($M+1$) with $m/z = 229$. Analytical reversed-phase HPLC analysis (see conditions for MG-H1, above) indicated the presence of only one compound, MG-H3, at the retention time of 1.9 min.

THP (Figure 1d)

This was prepared similarly to the N_α-acetyl-THP derivative [16]. Briefly, N_α-t-Boc-arginine (100 mM) was incubated with methylglyoxal (100 mM) in 100 mM sodium phosphate buffer, pH 7.4 and 37 °C (2 ml), for 14 days with adjustment of the pH to 7.4 with sodium hydroxide (5 M) as required. The product mixture was lyophilized to dryness, extracted with ethanol (once with 2.5 ml, twice with 1 ml) and ethanol removed from the combined extracts *in vacuo*. The residue was dissolved in 25 mM acetic acid with 15 % methanol (0.4 ml) and purified by preparative reversed-phase HPLC; sample loading was 0.2 ml. The eluent was 25 mM acetic acid with 15 % methanol, the flow rate was 9.9 ml/min and the eluate absorbance was monitored at 230 nm. N_α-t-Boc-THP eluted in the retention interval 6–9 min. The solvent was removed *in vacuo* and the residual solid re-chromatographed under similar conditions except that the eluent contained 10 % methanol; N_α-t-Boc-THP eluted in the retention interval 13–23 min. The solvent was removed *in vacuo*, the residual compound was de-protected by stirring in 0.5 M HCl (0.5 ml) overnight at room temperature and the sample lyophilized to dryness to yield 7.76 mg of solid (yield, 12 %). THP had the following characteristics. ^{1}H NMR (^2H_2O): pyrimidinyl 1.48 (s, 3H, 6-CH_3), 1.53 (s, 3H, 4-CH_3) and 3.75 (s, 1H, 5-H); ornithyl 1.65 (m, 2H, 4-H), 1.83 (m, 2H, 3-H), 3.25, $J_{4,5} = 7$ Hz (t, 2H, 5-H) and 4.14 (m, 1H, 2-H). MALDI-MS analysis gave a molecular ion ($M+1$) with $m/z = 319$. Analytical reversed-phase HPLC analysis (see conditions for MG-H1, above) indicated the presence of only one compound, THP, at a retention time of 1.4 min.

Preparation of glyoxal-derived hydroimidazolone, N_δ-(5-hydro-4-imidazolon-2-yl)-ornithine (G-H1; isomer 1), and 3-deoxyglucosone-derived hydroimidazolone, N_δ-[5-(2,3,4-trihydroxybutyl)-5-hydro-4-imidazolon-2-yl]ornithine (3DG-H1)

G-H1 was prepared by analogous methods to those used for the preparation of MG-H1, starting with glycine instead of alanine. The overall yield for the five steps was 4 %. G-H1 was characterized by ^{1}H NMR (270 MHz, [^{2}H]methanol). δ_H (p.p.m.) and J values were: hydroimidazolonyl 4.23 (s, 2H, 5-H); ornithyl

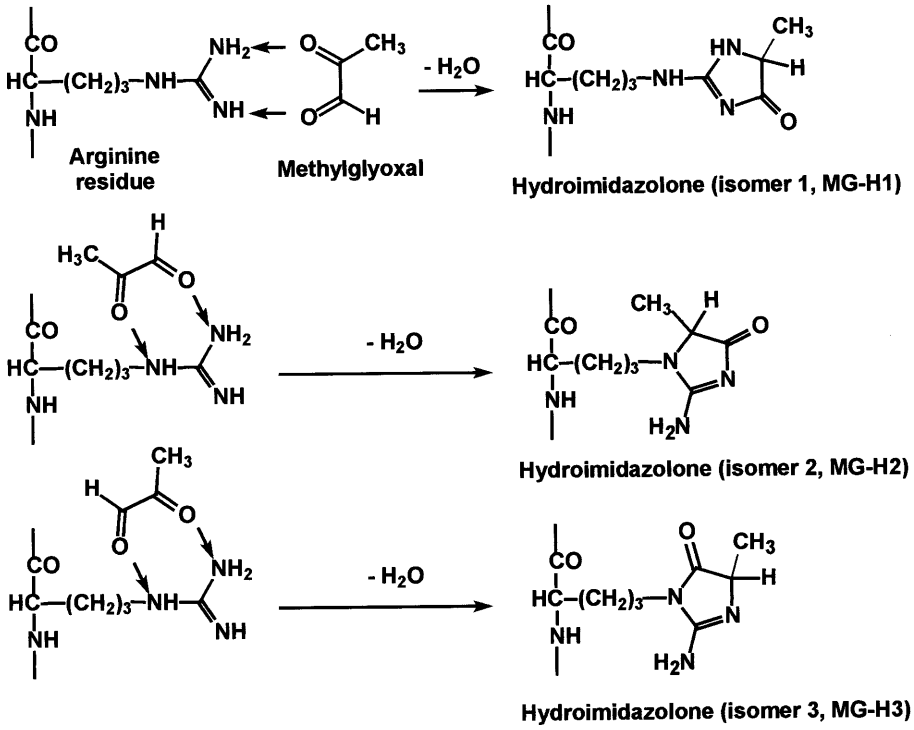

Figure 4 Structural isomerism of methylglyoxal-derived hydroimidazolone AGE residues formed from arginine residues and methylglyoxal in proteins

1.9–2.0 (m, 4H, 3-H and 4-H), 3.45 $J_{4,5} = 6.6$ (t, 2H, 5-H) and 4.06, $J_{2,3} = 5.7$ (t, 1H, 2-H). MALDI-MS analysis gave a molecular ion ($M+1$) with an m/z value of 215.

3DG-H1 was prepared by incubation of N_{α}-t-Boc-arginine (0.5 mmol, 155 mg) with 3-deoxyglucosone (0.55 mmol, 103 mg), which were dissolved in 2.5 ml of 1 M sodium phosphate buffer, pH 7.4, and incubated at 37 °C for 7 days under aseptic conditions. The product mixture was then lyophilized to dryness and residue extracted with methanol (once with 2.5 ml, twice with 1 ml). Methanol was removed from the combined extracts *in vacuo*. N_{α}-t-Boc-3DG-H1 was purified by preparative reversed-phase HPLC. The mobile phase was 0.1 % TFA in water with an elution profile of 10 % methanol for 0–10 min, and a linear gradient of 10–50 % methanol over 10–30 min. The flow rate was 9.9 ml/min. The sample loading was 30 mg in 0.3 ml of 0.1 % TFA in water. The eluate absorbance was monitored at 225 nm. Eluate corresponding to the absorbance peak in the retention interval 14–16 min was collected and lyophilized to dryness. The solid residue was dissolved in 0.5 M HCl (1.0 ml), stirred overnight at room temperature and lyophilized to give 3DG-H1. This was characterized by ^{1}H NMR (270 MHz, [^{2}H]methanol). δ_H and $J_{x,y}$ values were: hydroimidazolonyl 4.48 (m, 1H, 5-H); 2,3,4-trihydroxybutyl 1.98 (m, 1H, 1-H_A), 2.28 (m, 1H, 1-H_B), 3.50 (m, 1H, 3-H), 3.65 (m, 1H, 2-H), 3.68 (m, 1H, 4-H_A) and 3.75 (m, 1H, 4-H_B); ornithyl 1.7–1.9 (m, 4H, 3-H and 4-H), 3.39, $J_{4,5} = 6.8$ (t, 2H, 5-H) and 4.07 (m, 1H, 2-H). MALDI-MS gave a molecular ion ($M+1$) with an m/z value of 319. The yield was 7 mg (4 %).

Preparation of other glycation adducts and internal standard (IS)

The following glycation adducts were prepared by published methods: N_{ε}-(1-deoxy-D-fructos-1-yl)lysine (fructosyl-lysine) [10], CML [15], CEL [26], pyrraline [42], GOLD, MOLD [43], DOLD [29], pentosidine [44] and argpyrimidine [31]. 2-Ethylamino-4,6-dimethyl-5-hydroxypyrimidine, IS for the chromatographic assay of AGEs with intrinsic fluorescence, was prepared by dissolving ethylguanidine (696 mg, 8 mmol) in 400 mM methylglyoxal (20 ml) in water and lyophilization to dryness. Ethanol (2 ml) was added and the sample was left at room temperature overnight. The solvent was evaporated *in vacuo*, dissolved in methanol (1 ml) and precipitated with tetrahydrofuran (9 ml). The solid collected was dried *in vacuo* and purified by preparative reversed-phase HPLC. The mobile phase was 0.1 % TFA in water with a gradient of 0–100 % methanol over 20 min. The eluate was monitored by absorbance at 320 nm. The desired product was eluted in the retention interval 12–14 min; the retention fraction was lyophilized to dryness to give the product. ^{1}H NMR analysis gave δ_H and coupling constant $J_{x,y}$ (Hz) values of: 2-aminoethyl 1.21 (tr, $J_{1,2} = 7.3$, 3H, 2-H) and 3.42 (q, $J_{1,2} = 7.3$, 2H, 1-H); pyrimidinyl 2.43 (s, 6H, 4-CH_3 and 6-CH_3). MALDI-MS gave a molecular ion of ($M+1$) with $m/z = 168$. The yield was 94 mg (7 %).

Preparation of HSA with minimal and high contents of CML and CEL residues

HSA with a minimal content of CML (CML_{min}-HSA) was prepared by incubation of HSA (0.66 mM) with glyoxylic acid (2.15 mM) and sodium cyanoborohydride (56 mM) in 200 mM sodium phosphate buffer, pH 7.8, at 37 °C for 24 h under aseptic conditions. HSA with a high content of CML (CML-HSA) was prepared by incubation of HSA (0.66 mM) with glyoxylic acid (21.5 mM) and sodium cyanoborohydride (56 mM) in 200 mM sodium phosphate buffer, pH 7.8, at 37 °C for 24 h under aseptic conditions. HSA derivatives with minimal and high contents of CEL (CEL_{min}-HSA and CEL-HSA, respectively) were prepared by analogous methods using pyruvic acid instead of glyoxylic

acid. All proteins were then dialysed against PBS, pH 7.4 and 4 °C, and stored at −20 °C.

Chromatographic assay of glycation adducts in proteins by derivatization with AQC

Prior to AGE analysis, protein samples were washed by ultrafiltration (10 kDa cut-off membrane) and hydrolysed enzymically. An aliquot of protein sample (≈ 500 μg) was diluted to 500 μl with water and concentrated to ≈ 50 μl by ultrafiltration with the same membrane. This was repeated a further two times to complete the washing process. The protein concentration was then determined by the Bradford method [45]. An aliquot of sample (100 μg of protein) was diluted to 20 μl with water. Aliquots of 40 mM HCl (25 μl), pepsin solution (2 mg/ml in 20 mM HCl; 5 μl) and thymol solution (2 mg/ml in 20 mM HCl; 5 μl) were added and the sample was incubated at 37 °C for 24 h. The sample was then neutralized and buffered at pH 7.4 by addition of 25 μl of 0.5 M potassium phosphate buffer, pH 7.4, and 5 μl of 260 mM KOH. Fructosyl-lysine derivatives were susceptible to oxidative degradation to CML residues at neutral and high pH, so subsequent steps were performed under nitrogen to inhibit the degradation of fructosyl-lysine and overestimation of CML residues [14,46]. Pronase E solution (2 mg/ml in 10 mM potassium phosphate buffer, pH 7.4; 5 μl) was added and the sample was incubated at 37 °C for 24 h. Aminopeptidase and prolidase solutions (both 2 mg/ml in 10 mM potassium phosphate buffer, pH 7.4; 5 μl) were added and the sample incubated at 37 °C for 48 h. This gave the final enzymic hydrolysate (100 μl) for the AGE assay.

Aliquots of enzymic hydrolysate (50 μl, equivalent to 50 μg of protein) were placed in 1 ml glass vials and IS (α-aminobutyric acid, 100 nmol/ml; 10 μl) was added. Water (40 μl), AQC-derivatizing buffer [500 mM borate buffer/400 μM diethylenetriaminepenta-acetic acid (DETAPAC), pH 8.8; 100 μl] and AQC (10 mM in acetonitrile; 200 μl) were added. Calibration standards contained 0–1 nmol of AGEs and 0–2.5 nmol of fructosyl-lysine with 0–20 nmol of amino acids. Glycation adducts analysed were: G-H1, 3-DG-H1, MG-H1, MG-H2, MG-H3, THP, fructosyl-lysine, CML, DOLD, argpyrimidine, GOLD, MOLD, pyrraline, pentosidine and CEL. Amino acids analysed were: Ala, Arg, Asp, Glu, Gly, Phe, His, Ile, Lys, Leu, Met, Pro, Ser, Thr, Trp, Tyr and Val. Test and calibration standard samples were incubated at 55 °C for 10 min. The samples were then dried by centrifugal evaporation at room temperature, reconstituted in water (100 μl) and the precipitate removed by centrifugal filtration (0.2 μm). The filtrate was analysed for AGEs, fructosyl-lysine and amino acids by analytical reversed-phase HPLC.

HPLC analysis employed a custom three-solvent gradient adapted from van Wandelen and Cohen [47]. Changes were: (i) the extension of analysis time to 220 min, with 45 min washing and re-equilibration (an overall run time of 265 min; Table 1); (ii) increased sample loading equivalent to ≈ 150 nmol of amino acids; and (iii) detection by absorbance and fluorescence spectrophotometry for AQC-amino acid and AQC-AGE adduct detection, respectively.

Validation studies

The statistical variation, limit of detection (LOD; the analyte concentration equal to three times the S.D. of the zero analyte control), optimum sample processing, analyte and AQC-adduct stabilities during batch analysis and sample storage, analytical recoveries and interferences in the AQC-based chromatographic assay of AGEs were investigated. The effect of acid hydrolysis and enzymic hydrolysis of AGEs was investigated. Acid hydrolysis was performed by the incubation of 100 μg of protein in 6 M HCl with 0.1 % phenol and 0.05 % mercaptoethanol at 110 °C for 24 h. The effect of the addition of antioxidants to the sample processing (DETAPAC in the enzymic hydrolysis and AQC derivatization; enzymic hydrolysis under air and nitrogen), antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin) in the enzymic hydrolysate, amount of protease and peptidase on enzymic hydrolysis of the protein and amount of AQC on the derivatization were investigated.

For analytical recovery studies, authentic adducts were formed by the derivatization of fructosyl-lysine and AGEs by AQC, as described above, scaled up to 2.8 μmol of analyte. The AQC adduct was then purified by preparative reversed-phase HPLC. The eluent was 0.1 % TFA with a linear gradient of 0–30 % in methanol over 30 min. The eluate absorbance was monitored at 245 nm, and retention fractions collected. Fractions were lyophilized, reconstituted in 100 μl of water and analysed by MALDI-MS to identify the desired AQC adduct. AQC adducts were mono- or di-derivatized by AQC. AQC analyte/AQC IS peak-area ratios were compared for chromatographic analysis of derivatized samples relative to peak-area ratios from chromatographic analysis of authentic AQC-analyte and AQC-IS adducts over the range 50–1000 pmol of AGE or authentic AQC-AGE adduct.

Interferences in the AQC chromatographic analysis of AGEs were investigated by two procedures: (i) investigation of AQC derivatization and chromatographic analysis of trace amino acid derivatives present in physiological proteins and (ii) collection of retention eluate fraction from the analytical HPLC analysis and MALDI-MS of the de-salted sample. Compounds tested in procedure (i) were fructosyl-valine, nitrotyrosine, methionine sulphoxide and methionine sulphone. In procedure (ii), eluate samples were collected, lyophilized to dryness, reconstituted in water (1.0 ml) and eluted through an octadecyl silica (ODS)

Table 1 Chromatographic method for AGE analysis: HPLC solvent programme

Solvent compositions: solvent A, 140 mM sodium acetate and 17 mM triethylamine in water, adjusted to pH 5.05 with phosphoric acid; solvent B, 60% acetonitrile in water; solvent C, 140 mM sodium acetate and 17 mM triethylamine in water, adjusted to pH 6.8 with phosphoric acid. Flow rate, 1 ml/min. The gradient column refers to the change in solvent composition from the preceding row to the concurrent row. Part (b) represents a supplementary chromatographic elution programme for resolution of arginine and threonine.

(a)

Time (min)	Solvent ...	A (%)	B (%)	C (%)	Gradient
0		96	4	0	–
50		95	5	0	Linear
95		88	12	0	Linear
96		27	13	60	Linear
120		24	16	60	Linear
130		24	16	60	Linear
131		84	16	0	Linear
180		84	16	0	Linear
220		60	40	0	Linear
250		0	100	0	Immediate (washing)
265		96	4	0	Immediate (re-equilibration)
(b)					
0		95	5	0	–
50		95	5	0	Linear
95		88	12	0	Linear
125		0	100	0	Immediate (washing)
140		96	4	0	Immediate (re-equilibration)

solid-phase extraction cartridge (500 mg of ODS). The ODS cartridge was washed with 3 ml of water and then the AQC-analyte adduct eluted with 3 ml of methanol. The methanol was removed and the residual solid analysed by MALDI-MS.

Stability studies: stability of novel hydroimidazolone AGEs, AQC-AGE and AQC-amino acid adducts during analysis and in storage

The stability of the methylglyoxal-derived hydroimidazolone structural isomers, THP and argpyrimidine at 37 °C and pH 5.4–9.4 was investigated by reversed-phase HPLC analysis of individual AGEs with absorbance detection at 210 nm, (hydroimidazolones, THP and phenylalanine) or fluorescence with an excitation wavelength of 320 nm, and an emission wavelength of 382 nm, (argpyrimidine). The mobile phase was 10 mM sodium phosphate buffer, pH 7.4. The flow rate was 1 ml/min. Retention times were: hydroimidazolones, 1.9 min; THP, 3.52 min; IS (phenylalanine), 5.3 min; and argpyrimidine, 9.8 min. Hydroimidazolone, THP or argpyrimidine (1 nmol) with the IS (0.1 mg/ml) were incubated at 37 °C in 100 mM buffer (100 μl; pH 5.4, acetate buffer; pH 7.4, phosphate buffer; pH 9.4, pyrophosphate buffer) for 0–24 h. Aliquots (10 for each run) were withdrawn and analysed. Analyte/IS peak-area ratios were fitted to a first-order exponential decay and half-lives determined.

The stability of the AQC-derivatized analytical standards during batch analysis at 18 °C and at −20 °C in storage was investigated by repeated chromatographic analysis of derivatized samples over 0–8 days (five runs) and analysed. Analyte/IS peak-area ratios were fitted to a first-order exponential decay and the $t_{0.95}$ value, the time for 5% degradation of the AQC adduct, determined.

Chromatographic assay of AGEs with intrinsic fluorescence

AGEs with intrinsic fluorescence (argpyrimidine and pentosidine) were analysed in enzymic hydrolysates of proteins by analytical reversed-phase HPLC with fluorimetric detection without derivatization. Aliquots of enzymic hydrolysate (20 μl, equivalent to 20 μg of protein) were mixed with IS (2-ethylamino-4,6-dimethyl-5-hydroxypyrimidine, 5 nmol/ml; 10 μl) and water (20 μl). Calibration standards contained 1–75 pmol of pentosidine and 10–1000 pmol of argpyrimidine. Test and calibration standard were analysed by reversed-phase HPLC. The solvent was 10 mM sodium phosphate buffer with 10 mM *n*-heptafluorobutyric acid, pH 7.0, and a linear gradient of 0–30% methanol from 20–60 min. The eluate fluorescence was monitored at $\lambda_{max,excitation}/\lambda_{max,emission}$ of 320/385 nm. LOD values were 8.8 pmol for argpyrimidine and 1.4 pmol for pentosidine.

RESULTS

Preparation and characterization of methylglyoxal-derived hydroimidazolone derivatives of arginine and other AGEs

The participation of all three guanidino nitrogen atoms in the formation of hydroimidazolone derivatives formed by the reaction of methylglyoxal with L-arginine is expected (Figure 4). This leads to the formation of three structural isomers, each of which exists in two epimeric forms due to racemization in the hydroimidazolone ring. Routes were devised for the unambiguous preparation of two of the structural isomers, MG-H1 and MG-H2, and we isolated the third isomer from the structural isomeric mixture formed from the reaction of methylglyoxal with N_α-t-Boc-arginine.

Reversed-phase HPLC analysis of MG-H1 after derivatization with AQC, with IS α-aminobutyric acid, indicated the presence of two peaks with similar retention times and peak areas, 79.7 and 82.0 min (Figure 5a). The two compounds resolved in the chromatogram had identical MALDI-MS spectra, giving a molecular ion $(M+1)$ of $m/z = 399$. These were assigned to equal amounts of the two epimers of MG-H1, with racemization of the chiral centre at C_5 of the hydroimidazolone ring. Attempts to prepare the hydroimidazolones stereoselectively from L- and D-alanine starting materials failed and gave identical chromatographic peaks, as shown. Similarly, HPLC analysis of AQC-derivatized MG-H2 gave two peaks with retention times of 84.1 and 89.7 min (Figure 5b) and a molecular ion $(M+1)$ of $m/z =$ 399, and HPLC analysis of AQC-derivatized MG-H3 gave two

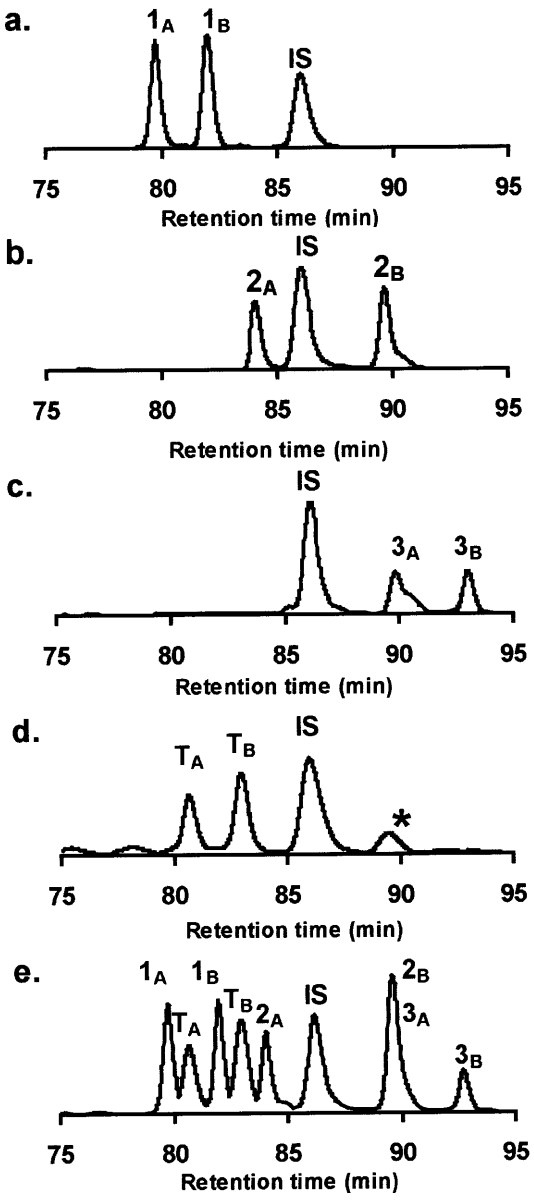

Figure 5 HPLC analysis of MG-derived hydroimidazolones after derivatization with AQC

(**a**) Two epimeric forms of MG-H1 (1_A and 1_B) with retention times of 79.7 and 82.0 min, with IS (retention time 86.0 min). (**b**) Two epimeric forms of MG-H2 (2_A and 2_B) with retention times of 84.1 and 89.7 min, with IS. (**c**) Two epimeric forms of MG-H3 (3_A and 3_B) with retention times of 89.9 and 93.0 min, with IS. (**d**) Two epimeric forms of THP (T_A and T_B) with retention times of 80.8 and 83.0 min, with IS. * indicates the AQC degradation product. (**e**) Epimeric forms of all three hydroimidazolones and THP with IS.

Table 2 Stability of methylglyoxal-derived AGEs at 37 °C

ND, not determined.

AGE	Half-life (days)		
	pH 5.4	pH 7.4	pH 9.4
MG-H1	16.1 ± 4.3	11.7 ± 2.7	0.87 ± 0.15
MG-H2	10.1 ± 2.1	6.3 ± 1.3	2.12 ± 0.31
MG-H3	14.8 ± 2.9	0.66 ± 0.05	0.013 ± 0.001
THP	ND	1.8 ± 0.03	ND
Argpyrimidine	ND	1.66 ± 0.22	ND
Argpyrimidine (+100 μM DETAPAC)	ND	9.3 ± 2.8	ND

peaks with retention times of 89.9 and 93.0 min (Figure 5c) and a molecular ion $(M+1)$ of $m/z = 399$. These were assigned to the two epimers of MG-H2 and MG-H3, respectively. Reversed-phase HPLC analysis of THP after derivatization with AQC indicated the presence of two peaks with similar retention times and peak areas; 80.8 and 83.0 min (Figure 5d). The two compounds resolved in the chromatogram had identical MALDI-MS spectra, giving a molecular ion $(M+1)$ with $m/z = 489$. These were assigned to the two epimers of THP. There are expected to be eight epimers of THP but they were resolved only into two groups. Chromatographic analysis of all three structural isomers of methylglyoxal-derived hydroimidazolones and THP derivatized with AQC gave a chromatographic peak fingerprint consistent with the six epimers (Figure 5e). The hydroimidazolone structural isomers and THP or their AQC adduct did not undergo structural isomerization during the analysis.

The stability of the free hydroimidazolone isomers, THP and argpyrimidine was studied at 37 °C (Table 2). The stability of methylglyoxal-derived hydroimidazolones decreased with increasing pH; the stability of MG-H3 decreased markedly with an increase in pH. THP and argpyrimidine were also unstable at pH 7.4 and 37 °C, although the half-life of argpyrimidine was increased in the presence of the metal-ion chelator DETAPAC.

Hydroimidazolone derivatives derived from glyoxal (G-H1) and 3-deoxyglucosone (3DG-H1) were prepared. The AQC adduct of these AGEs eluted with retention times of 61.0 and 66.5 min, and had molecular ions $(M+1)$ with m/z values of 385 and 489, respectively.

Chromatographic assay of fructosyl-lysine and AGEs by derivatization with AQC and reversed-phase HPLC with fluorimetric detection

The AQC method for the derivatization of amino acids [47] was applied to the assay of glycation adducts in proteins. Analytes

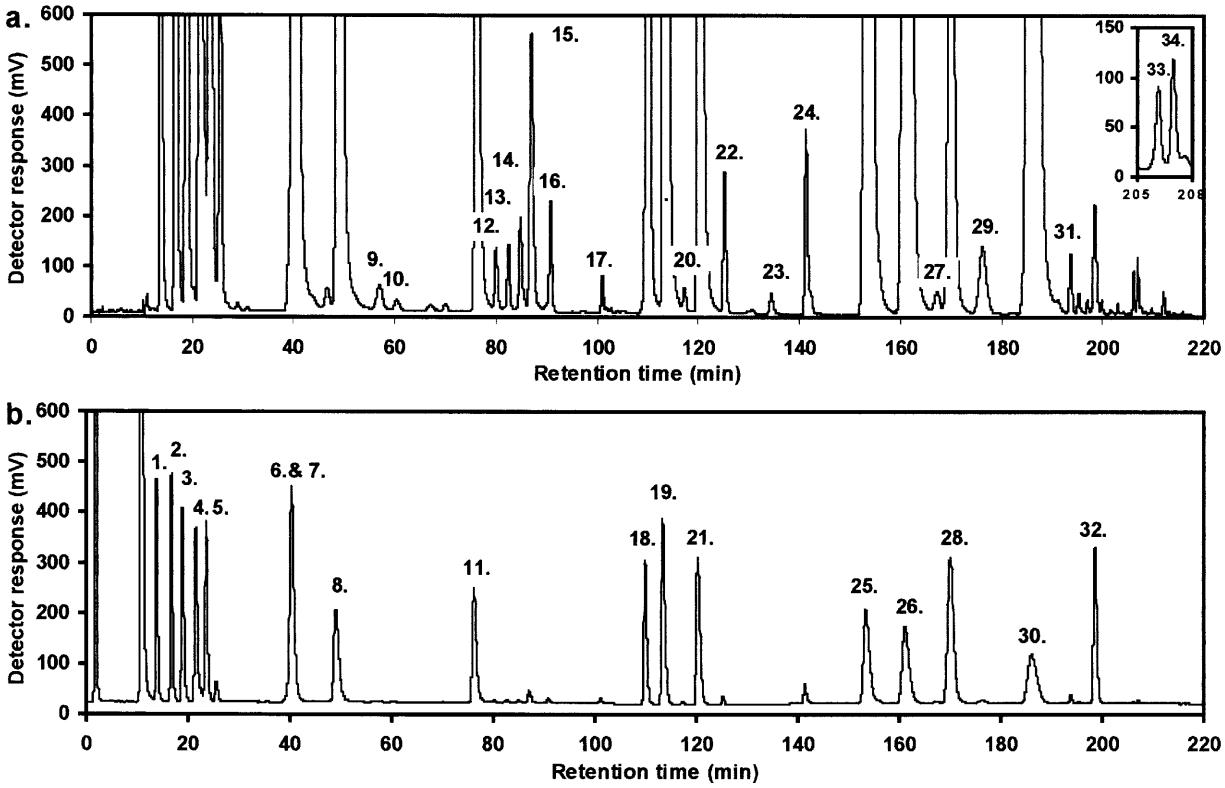

Figure 6 Chromatographic assay of fructosyl-lysine, AGEs and amino acids by pre-column derivatization with AQC and (a) fluorescence and (b) absorbance detection

Chromatograms: calibration standard of fructosyl-lysine and AGEs (1 nmol) with amino acids (20 nmol). The following key gives retention time (min) and analyte: peak 1, 14.0, Asp; peak 2, 17.3, Ser; peak 3, 19.1, Glu; peak 4, 22.1, Gly; peak 5, 24.5, His; peaks 6 and 7, 41.7, Arg and Thr; peak 8, 50.1, Ala; peak 9, 61.0, G-H1; peak 10, 66.5, 3DG-H1; peak 11, 77.4, Pro; peak 12, 82.8, MG-$H1_A$; peak 13, 85.1, MG-$H1_B$; peak 14, 86.8, MG-$H2_A$; peak 15, 89.3, IS (α-aminobutyric acid); peak 16, 94.1, MG-$H2_B$ and AQC degradation product; peak 17, 101.1, fructosyl-lysine; peak 18, 112.4, Tyr; peak 19, 115.5, Val; peak 20, 117.5, CML; peak 21, 122.6, Met; peak 22, 125.3, DOLD; peak 23, 134.5, argpyrimidine; peak 24, 140.9, GOLD; peak 25, 155.3, Ile; peak 26, 163.1, Leu; peak 27, 166.1, MOLD; peak 28, 173.5, Lys; peak 29, 175.6, pyrraline; peak 30, 188.4, Phe; peak 31, 193.3, pentosidine; peak 32, 202.1, Trp; peak 33, 205.6, CEL_A; peak 34, 206.5, CEL_B.

Table 3 Characteristics of AQC adducts of fructosyl-lysine, AGEs and analytes

All AQC-amino acid adducts other than those shown had $t_{0.95}$ values $>$ 14 days, except His (5.3 $\pm$ 0.4 days) and Met (6.3 $\pm$ 0.6 days). $t_{0.95}$ values were calculated at 18 °C.The ratios in parentheses in the second column indicate the AGE/AQC ratio. c.v., coefficient of variance.

Analyte	Molecular ion of AQC adduct (Da)	AQC-adduct stability $t_{0.95}$ (days)	Recovery (%)	LOD (pmol)	Intrabatch c.v. (%)	Interbatch c.v. (%)
G-H1	$M+1$ = 385 (1:1)	41 $\pm$ 7	85	14	7	17
3DG-H1	$M+1$ = 489 (1:1)	23 $\pm$ 7	99	8	17	34
MG-H1	$M+1$ = 399 (1:1)	15 $\pm$ 5	95	2	4	7
MG-H2	$M+1$ = 399 (1:1)	60 $\pm$ 19	96	5	5	5
THP	$M+1$ = 319 (1:1)	0.67 $\pm$ 0.01	96	2	5	7
Fructosyl-lysine	$M+1$ = 649 (1:2)	12 $\pm$ 5	90	14	10	18
CML	$M+1$ = 545 (1:2)	0.78 $\pm$ 0.11	93	12	5	9
DOLD	M = 771 (1:2)	28 $\pm$ 5	50	7	4	6
Argpyrimidine	$M+1$ = 425 (1:1)	0.2 $\pm$ 0.03	81	17	3	4
GOLD	M = 667 (1:2)	6.3 $\pm$ 0.1	89	4	13	18
MOLD	M = 681 (1:2)	1.1 $\pm$ 0.4	50	11	4	24
Pyrraline	$M+1$ = 425 (1:1)	23 $\pm$ 1	95	8	2	29
Pentosidine	M = 719 (1:2)	6.8 $\pm$ 0.7	55	12	4	4
CEL	$M+1$ = 559 (1:2)	3.4 $\pm$ 0.7	75	17	4	5

were resolved by reversed-phase HPLC and detected by absorbance and fluorescence. Specimen chromatograms for fluorescence and absorbance detection are given in Figure 6. G-H1 and 3DG-H1 (peaks 9 and 10) eluted between Ala and Pro (peaks 8 and 11). MG-H1 epimers A and B and MG-H2 epimer A (peaks 12–14) eluted between Pro and the IS (peak 15). MG-H2 epimer B and fructosyl-lysine eluted between the IS and Tyr (peak 18). CML (peak 20) eluted between Val (peak 19) and Met (peak 21). DOLD, argpyrimidine and GOLD (peaks 22–24) eluted between Met and Ile (peak 25). MOLD (peak 27) eluted between Leu and Lys (peaks 26 and 28), and pyrraline (peak 29) between Lys and Phe (peak 30). Pentosidine (peak 31) eluted between Phe and Trp (peak 32) and epimers A and B of CEL (peaks 33 and 34) eluted after Trp. It was difficult to resolve Arg from Thr in the chromatographic run without losing resolution of other amino acids and/or AGEs. Arg and Thr were resolved in a second chromatographic run of 95 min where a slightly steeper acetonitrile gradient decreased the retention times of Arg and Thr but decreased that of Arg more markedly and resolved these analytes. The molecular characteristics of the AQC adducts were investigated (Table 3). Some AGEs were mono-derivatized by AQC (hydroimidazolones, THP, argpyrimidine and pyrraline) and fructosyl-lysine and other AGEs (GOLD, MOLD, DOLD, pentosidine, CML and CEL) were di-derivatized. The AQC adducts of amino acids and AGEs were generally stable; $<$ 5% degradation occurred in storage over 2 weeks in storage at $-$20 °C and over 24 h at 18 °C in the autosampler. The exceptions to this were AQC adducts of MG-H3, argpyrimidine, THP and CML. AQC-MG-H3 degraded markedly during a single analytical run at 18 °C ($t_{1/2}$ = 3.3 h), and hence was not quantified. AQC-argpyrimidine was stable for 2 weeks in storage at $-$20 °C but degraded with a half-life of 2.7 $\pm$ 0.4 days at the autosampler temperature of 18 °C ($\approx$ 5% degradation in 5 h). There was, therefore, a loss of $\approx$ 27% of argpyrimidine-AQC adduct over a 24 h analysis sample batch. AQC-THP and AQC-CML adducts were stable in storage at $-$20 °C and were more stable than AQC-argpyrimidine at 18 °C: $\approx$ 7 and 6% of these adducts, respectively, were lost over 24 h. Argpyrimidine, THP and CML were therefore included in the assay for quantitation. The analytical recoveries were 50–99%. Calibration curves of amino acids (0–20 nmol) and AGEs (0–1 nmol) were constructed and LOD values of AGEs deduced. LODs were 2–20 pmol and the interbatch coefficients of variation were 4–34%, depending on the analyte. Generally, AQC adducts of AGEs were quantified by fluorescence and amino acids by absorbance. Absorbance detection was employed for some AGE-AQC adducts; however, particularly AQC adducts of CML, argpyrimidine, DOLD, GOLD, MOLD and pentosidine.

Table 4 AGE content of HSA modified minimally and highly to form CML and CEL residues

Data are means $\pm$ S.D. from three determinations.

Protein	CML content (mol/mol of protein)	CEL content (mol/mol of protein)
HSA	$<$ 0.03	$<$ 0.05
CML_{min}-HSA	1.35 $\pm$ 0.05	$<$ 0.05
CML-HSA	10.7 $\pm$ 0.6	$<$ 0.05
CEL_{min}-HSA	$<$ 0.03	1.01 $\pm$ 0.05
CEL-HSA	$<$ 0.03	23.5 $\pm$ 0.8

For the analysis of amino acids and AGEs in proteins glycated *in vitro* and *in vivo* by the AQC-derivatization method, enzymic hydrolysis was used since acid hydrolysis led to $>$ 90% loss in hydroimidazolone AGEs. Enzymic hydrolysis was performed with pepsin at pH 1.7 and then with pronase E, prolidase and aminopeptidase at pH 7.4. The steps at pH 7.4 were performed under nitrogen to inhibit oxidative processes. Enzymic hydrolysis under aerobic conditions overestimated CML, GOLD and G-H1 and underestimated fructosyl-lysine. Enzymic hydrolysates equivalent to 25 μg of protein were used in the assay of glycation adducts. Higher sample loading gave decreased resolution of analytes. Other modifications to the AQC method were (i) inclusion of antibiotics in the enzymic hydrolysis procedure after the pepsin step to prevent bacterial consumption of amino acids and (ii) addition of the metal-ion chelator DETAPAC in the AQC derivatization buffer to decrease the oxidative degradation of glycation adducts during derivatization. DETAPAC was not included in the post-pepsin hydrolysis steps since it inhibited the proteases.

To demonstrate the utility of the AQC assay for AGEs, HSA that had been modified minimally and highly by CML and CEL was analysed (Table 4). Retention intervals for chromatograms

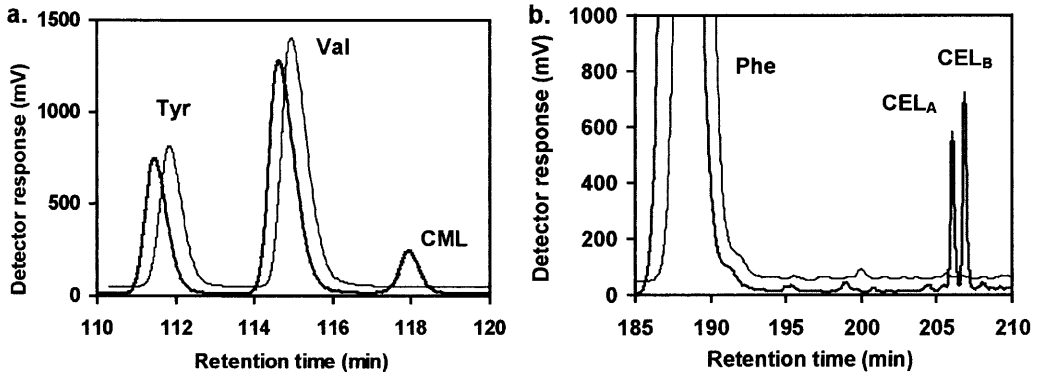

Figure 7 Detection of AGEs in HSA modified specifically to form CML and CEL residues

Retention intervals of the chromatograms of (**a**) HSA and CML-HSA (absorbance detection) and (**b**) HSA and CEL_{min}-HSA (fluorescence detection) by the AQC method of chromatographic analysis of AGEs. Thick lines, modified HSA; thin lines, unmodified HSA. In each graph, the two sets of lines are displaced on the *x* and *y* axes for clarity.

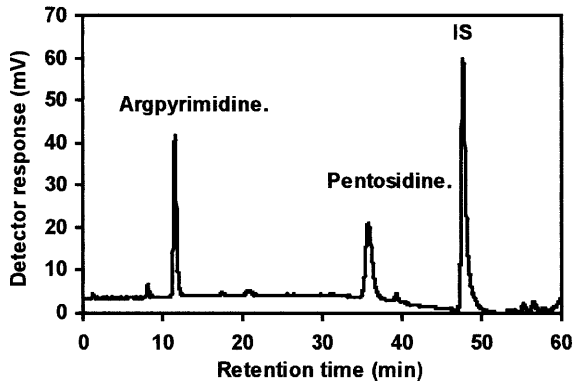

Figure 8 Chromatographic assay of AGEs with intrinsic fluorescence: argpyrimidine and pentosidine

A specimen chromatogram is shown. Argpyrimidine (50 pmol), pentosidine (5 pmol) and IS (50 pmol).

of CML-HSA (absorbance detection) and CEL_{min}-HSA (fluorescence detection), with HSA control, are given in Figure 7. The AQC-CML peak was detected at a retention time of $\approx$ 118 min in CML_{min}-HSA and CML-HSA and the AQC-CEL_A and AQC-CEL_B epimer peaks were detected at retention times of $\approx$ 207 and 208 min, respectively, in CEL_{min}-HSA and CEL-HSA. There was a significant difference in the contents of AQC-CEL_A and AQC-CEL_B, being 45 and 55% of total AQC-CEL, respectively.

Assay of AGEs in enzymic hydrolysates with intrinsic fluorescence

Assay of argpyrimidine and pentosidine in enzymic hydrolysates without AQC derivatization by reversed-phase HPLC with fluorimetric detection gave an analytical method more specific for these AGEs and increased sensitivity (Figure 8). Resolution and LOD values of the analytes were improved relative to the AQC method.

DISCUSSION

AGEs with different chemical structures and physiological functions are present in the same protein glycated *in vitro* and *in vivo* [1,32]. This gave us the impetus to develop assays to measure many different AGEs. This provides a survey of different AGEs in proteins to enable key AGE epitopes associated with specific functional activities to be identified and quantified [48,49]. To date, most studies on glycation have measured only one or two AGEs [15,25,26,28,35–38]. Immunochemical techniques used in the assay of AGEs have provided important analytical evidence for the presence and ultrastructural location of AGEs but epitope-recognition characteristics of the antibodies used have often been unknown or known incompletely [38,48]. This is the first attempt to develop an assay that provides a comprehensive survey of AGEs, at least in proteins glycated *in vitro* where there is sufficient AGE content for the AQC method to be applied successfully.

In recent years, α-oxoaldehydes were identified as key intermediates in the glycation of proteins to form AGEs *in vitro* and *in vivo* [2,4–6,16,50,51]. The importance of arginine as a site of glycation in proteins has also been recognized and hydroimidazolones are important arginine-derived AGEs. Hydroimidazolones are formed by the reaction of arginine residues with α-oxoaldehydes, particularly methylglyoxal, glyoxal and 3-deoxyglucosone [6,22,25,51,52]. In this study, we also provided evidence of hydroimidazolone structural and stereochemical isomerism.

Inspection of the interaction of the α-oxoaldehydes with the guanidino N_ω group suggested that structural isomers of hydroimidazolones were available (Figure 3). Therefore, a complete description of the glycation of proteins by α-oxoaldehydes could be achieved only by studying all of these isomers. A suggestion of isomeric hydroimidazolones in glycated proteins was made previously [52] but the structures were unknown. The preparation of structural isomers of methylglyoxal-derived hydroimidazolones of arginine by unambiguous synthesis was preferred to enable unequivocal assignment of spectroscopic and chromatographic properties. Two isomers were prepared by unambiguous synthesis, MG-H1 and MG-H2. The third isomer, MG-H3, was not prepared in this way, but rather was isolated from the product mixture of the reaction of methylglyoxal with N_α-t-Boc-arginine and methylglyoxal; this incubation also produced MG-H1 but very little MG-H2. Several different synthetic routes for unambiguous synthesis of MG-H3 were tried but all failed. These procedures were successful for synthetic hydroimidazolones (without the ornithyl group).

MG-H1 was prepared conveniently by addition of the preformed hydroimidazolone ring to N_α-t-Boc-ornithine. Attempts were made to prepare L-arginine-derived epimers of MG-H1,

N_{δ}-L-(5-hydro-5-methyl-4-imidazolon-2-yl)-L-ornithine and N_{δ}-D-(5-hydro-5-methyl-4-imidazolon-2-yl)-L-ornithine, starting with L- or D-alanine. Chromatographic analysis, however, revealed a 1:1 epimer composition in the product. A stereochemically pure MG-H1 product was not achievable because of rapid epimerization. The epimerization of hydroimidazolones was also indicated by the loss of the hydroimidazolonyl 5-proton in ^2H_2O observed in ^1H NMR studies, as we and other investigators [22] have noted. The epimers were resolved by reversed-phase HPLC after derivatization with AQC. Similar epimers were found for the AQC adduct of CEL, which was double-derivatized with AQC. Derivatization of the structural and epimeric isomers of methylglyoxal-derived hydroimidazolones gave characteristic chromatographic peak pairs for each structural isomer. MG-H2 gave a distinctive ^1H NMR spectrum. The N_{δ} protons of the ornithyl residue were magnetically inequivalent and showed geminal and vicinal proton couplings (Figure 3). Oya et al. [16] detected a further arginine adduct formed from methylglyoxal, THP. This also gave two AQC adducts resolved in reversed-phase HPLC. Hence, the AQC derivatization method gave a chromatographic 'fingerprint' of methylglyoxal arginine adducts (except for argpyrimidine) in this region of the chromatogram.

The stability of the methylglyoxal-derived hydroimidazolone isomers generally decreased with increasing pH and each structural isomer had a distinctive stability and half-life. The hydroimidazolone half-life increased in the order MG-H1 > MG-H2 $\gg$ MG-H3, except at pH 5.4 where MG-H3 was more stable than MG-H2. At pH 7.4 and 37 °C, the half-life of MG-H1 was $\approx$ 12 days. This suggests that hydroimidazolones, unlike CML and CEL, are not likely to show a time-dependent accumulation in long-lived proteins *in vivo* over a time scale of years where protein turnover affects the AGE accumulation rate [53]. Rather, hydroimidazolones may reflect shorter-term periods of excessive glycation following periods of abnormal accumulation of precursor α-oxoaldehydes. An unexpected finding in the stability of methylglyoxal-derived AGEs was the short lifetime of THP and argpyrimidine (1.8 and 1.7 days, respectively, at pH 7.4 and 37 °C), although the lifetime of argpyrimidine was extended to 9.3 days under antioxidant conditions. Argpyrimidine was a major degradation product of THP. The identity of the hydroimidazolone degradation products was not determined herein but besides reversal of the hydroimidazolone formation reaction, probable degradation products are N_{ω}-(1-carboxyethyl)arginine. The closely related structure, N_{ω}-(1-carboxymethyl)arginine, formed from glyoxal, has been found in collagen [54]; both glyoxal-derived and methylglyoxal-derived hydroimidazolones were found in collagen [55].

Glyoxal- and 3-deoxyglucosone-derived hydroimidazolones, isomers 1, were also prepared and characterized. Glyoxal-derived hydroimidazolone isomer 2 was also prepared; its AQC adduct co-eluted with that G-H1 (results not shown). Preparation and resolution of structural isomers of glyoxal- and 3-deoxyglucosone-derived hydroimidazolones was not investigated further because the chromatographic run time was already long and the elution profile was complex. The 3DG-H1 AQC adduct eluted just after GH-1 in the AQC chromatographic assay of AGEs.

Development of the AQC chromatographic assay of AGEs

Derivatization of amino acids with AQC and chromatographic assay of the AQC adducts with fluorescence detection is an amino acid analysis technique that has high sensitivity and employs relatively mild derivatization conditions (pH 8.8, 55 °C for 10 min). This was developed for the assay of AGEs. Analytes with intrinsic fluorescence, tryptophan, pentosidine and argpyrimidine, gave low fluorescent emissions which was attributed to fluorescence quenching of the aminoquinolyl fluorophore [39]. It was essential to remove the excess AQC degradation products from the derivatized sample prior to chromatographic analysis, otherwise the chromatographic resolution was impaired severely. Analysis of this solid indicated the presence of AQC degradation products and $< 1\,\%$ of the AQC analytes. Validation studies of the assay gave interbatch variation and recoveries that would permit effective deployment of the AQC assay to quantify protein AGEs. Fructosyl-lysine, arginine and lysine content of protein samples were also routinely determined, and analytical data on other amino acids susceptible to glycation (N-terminal aspartate residue of albumin and β- and α-N-terminal valine residues of haemoglobin, for example) and oxidation (methionine, histidine and others, but not cysteine) were available. The retention time of nitrotyrosine was investigated. This analyte eluted just after phenylalanine and before pentosidine. The sensitivity was insufficient for detection of this analyte in samples *in vivo*, however.

A comparison was made between recoveries of hydroimidazolones in enzymic and acid hydrolysis of protein substrate. With acid hydrolysis, recoveries were $< 10\,\%$. This is in agreement with the chemical instability of hydroimidazolones under acid hydrolysis conditions [44,55]. Enzymic hydrolysis was used in all subsequent studies. Lost of hydroimidazolone during acid hydrolysis is probably the major reason why the presence of these adducts in proteins has been overlooked until recently.

The AQC chromatographic assay was deployed successfully for the quantification of CML and CEL in CML- and CEL-modified HSA prepared under conditions that gave proteins modified highly and minimally by the AGE; minimal modification having $\approx$ 1 mol and high modification having $\approx$ 10 mol of CML or CEL/mol of protein. Previous studies by other investigators used BSA with 18 mol of CML/mol of protein and ovalbumin with 6.2 and 0.66 mol of CML/mol of protein to investigate epitope recognition by the receptor for AGEs (RAGE) [56]. We have used CML-HSA and CML_{min}-HSA in similar studies [57].

The assay of AGEs by intrinsic fluorescence was developed for comparison with the AQC method. It was simple, and had greater sensitivity and specificity than the AQC method for argpyrimidine and pentosidine. Since pentosidine concentrations in plasma protein of normal healthy controls *in vivo* were very low, however, a larger protein sample than we used routinely was hydrolysed for its detection. This method had good agreement with the AQC method for relatively high amounts of argpyrimidine. When argpyrimidine content was extraordinarily high, as in highly methylglyoxal-modified HSA [40], absorbance detection was used.

The LOD values for AGE analytes, while better than absorbance-based amino acid detection procedures, were too high to detect AGEs in plasma protein *in vivo* in all but samples with extraordinarily high contents of AGEs. Increasing the sample loading above 25 μg of protein equivalents decreased the chromatographic resolution. Particularly for samples *in vivo*, chromatographic peaks of analytes were frequently not well resolved and hence confidence of discrete measurement of AGEs was in doubt. The detection *in vivo* of GOLD, MOLD, DOLD, CML, CEL and pyrraline was not possible because of sensitivity and/or poor chromatographic resolution. The AQC assay was particularly useful in applications for the measurement of proteins glycated *in vitro*, to minimal and high extents, and this is the application where it may find the most use. It was also useful for the resolution and detection of methylglyoxal-derived hydroimidazolone structural isomers, MG-H1 and MG-H2, and THP.

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