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Validation of a Reversed-Phase HPLC Method for Quantitative Amino Acid Analysis

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A semi-automated method for amino acid derivatization and analysis has been validated for use in analysis of protein biopharmaceuticals. The method includes protein hydrolysis, *o*-phthalaldehyde derivatization, and reversed-phase high-performance liquid chromatography analysis in a general-purpose UV-visible high-performance liquid chromatography system. Amino-acid derivatization is performed automatically by the high-performance liquid chromatography autosampler right before injection. The required validation parameters, i.e., specificity, linearity, accuracy, precision, limit of detection, and limit of quantification, were studied for bovine serum albumin and for a recombinant human Fab fragment. The method can be employed as an absolute quantification method for determination of extinction coefficients of recombinant proteins.

Key Words: Amino acid analysis, OPA-derivatization, reverse-phase HPLC, validation.

Amino-acid analysis has a long history in the characterization of protein-based products, since it provides information on the product concentration without referring to an external protein standard and it is independent from the shape and the charge of the protein. In addition, the determined amino-acid composition can confirm sample identity and gives a measure of sample purity. Furthermore, when combined with absorbance measurements, it allows the determination of extinction coefficients under various conditions.[1] For protein conjugates, where the synthetic counterpart modifies the protein absorption properties, amino-acid analysis may be required as the only reliable quantification method.

However, in spite of these features, few laboratories can perform such analysis in a reliable and quantitative way, due to the need for specialized equipment and skills. Usually, techniques based on ion-exchange separation coupled with post-column derivatization (e.g., with ninhydrin, the "classical" method) are considered more precise[1] than those based on pre-column derivatization and reversed-phase high-performance liquid chromatography (RP-HPLC), because the latter techniques imply extensive sample manipulation before analysis and are affected by the limited stability of the preformed derivatives.[2] However, such RP-HPLC-based methods have the advantage of being accessible to most analytical laboratories, since they do not require expensive dedicated instruments. In addition, manufacturing of dedicated instruments is being halted, making the availability of validated pre-column methods even more important.

In this paper, we describe the validation of a method that takes advantage of robotic sample derivatization, thereby limiting considerably the manual manipulation of samples. Another advantage of automation is that derivatization is performed just before the injection; therefore, the time from reaction to injection is kept absolutely constant for all samples, thus avoiding differential degradation of labile derivatives. We have studied the performance characteristics in terms of specificity, linearity, accuracy, precision, limit of detection, and limit of quantification for bovine serum albumin (BSA) and for a recombinant human Fab (rFab) fragment, whose extinction coefficient needs to be determined.

Protein samples were hydrolyzed, then automatically derivatized with *o*-phthalaldehyde (OPA) and in-line analyzed by RP-HPLC with ultraviolet-visible (UV-Vis) detection, according to a method published in an Agilent application note.[3]

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MATERIALS AND METHODS

Reagents, Solvents, and Materials

Sodium phosphate monobasic monohydrate, sodium hydroxide, boric acid, acetonitrile (LC grade), and methanol (LC grade) were obtained from Merck KGaA (Darmstadt, Germany). OPA reagent was prepared as described (Agilent art. 5061-3335, Palo Alto, CA). Borate buffer was prepared by adjusting 0.4 N boric acid to pH 10.2 with NaOH. Constant-boiling HCl was obtained from Sigma-Aldrich (St. Louis, MO). Chromatographic-grade water was produced by a Milli-Q system (Millipore, Billerica, MA)

Disposable glass test tubes (50 × 6 mm) and hydrolysis reaction vials (25 × 120 mm) with Mininert valves were from Kimble Glass, Inc., and Kontes Glass Co. (Vineland, NJ). Amber wide-opening vials, glass conical inserts with polymer feet, and screw caps were from Agilent.

Albumin standard solution (2 mg/mL) was supplied by Pierce Biotechnology (Rockford, IL), while amino acid standard mixtures at the concentration of 1 nmol/μL and 250 pmol/μL were from Agilent. The internal standard L-norvaline was obtained from Sigma-Aldrich. A recombinant Fab fragment (rFab) was obtained from the research laboratories of Bracco Imaging (Milan, Italy).

Amino Acid Standard Solutions

Amino acid standard samples were prepared by mixing 95 μL of the 250 pmol/μL amino acid standard mixture with 5 μL of 10 mM norvaline and analyzed directly by RP-HPLC, within 24 h from preparation. Solutions for linearity study were prepared in duplicate by diluting the 1 nmol/μL amino acid standard solution, and contained 20, 50, 130, 250, or 500 pmol/μL of amino acid standard mixture together with 0.5 mM norvaline.

Protein Samples

Glass test tubes (50 × 6 mm) were marked with incisions and soaked in a detergent solution for at least 12 h. They were rinsed thoroughly in Milli-Q water and dried in an oven at 80°C. Protein samples (7–75 μg) were transferred into the glass test tubes and spiked with 0.5 mM norvaline. They were quickly spun in a low-velocity centrifuge, then frozen and dried in a lyophilizer. Samples were then transferred into the reaction vial containing 0.5 mL of constant-boiling HCl on the bottom. Up to 12 test tubes could be accommodated in a reaction vial. The reaction vial was tightly closed and transferred into a pre-heated oven at 110°C for 18 h. The reaction vial was cooled at room temperature, then carefully opened under an aspirated hood. The test tubes were centrifuged and dried again in the lyophilizer to remove any liquid traces (condensed vapors). The dried residues were dissolved in 100 μL of 0.1 N HCl and transferred into the HPLC glass insert vials.

Instrumentation

Analyses were performed using an Agilent 1100 Liquid Chromatograph, equipped with a binary pump delivery system (G1312A), robotic autosampler (G1313A), column thermostat (G1316A) and multi-wavelength detector (G1365A).

Analytical Procedure

Chromatography conditions were in accordance with the Agilent method.[2] Briefly, the hydrolyzed samples and the norvaline-spiked amino acid standard solutions were automatically derivatized with OPA by programming the robotic autosampler (Table 1). After derivatization, an amount equivalent to 2.5 μL of each sample was injected on a Zorbax Eclipse-AAA column, 5 μm, 150 × 4.6 mm (Agilent), at 40°C, with detection at $\lambda = 338$ nm. Mobile phase A was 40 mM NaH_2PO_4, adjusted to pH 7.8 with NaOH, while mobile phase B was acetonitrile/methanol/water (45/45/10 v/v/v). The separation was obtained at a flow rate of 2 mL/min with a gradient program that allowed for 1.9 min at 0% B followed by a 16.3-min step that raised eluent B to 53%. Then washing at 100% B and equilibration at 0% B was performed in a total analysis time of 26 min.

RESULTS AND DISCUSSION

Acid hydrolysis is a crucial step that considerably influences amino-acid recovery. In fact, during acid hydrolysis, tryptophan and cysteine are destroyed and serine and threonine are also partially lost, while methionine can undergo oxidation. Moreover, some amino acids such as glycine and serine are common contaminants; therefore, their quantification needs careful subtraction of average responses in blank runs, which, in the case of glycine, is also complicated by the fact that this residue is known to give rise to multiple derivatives after OPA reaction.[2] Therefore, the validation parameters were estimated using the following seven best-recovered amino acids: Asx (Asn+Asp), Glx (Glu+Gln), Arg, Ala, Phe, Leu, and Lys.[4]

In order to fully assess the method's performance, both a standard amino acid mixture and a reference protein (e.g., BSA) should be assayed along with the product. The standard amino acid mixture (Figure 1) enables the verification of the HPLC method's performance, including derivatization, while the reference protein samples

TABLE 1

Autosampler Programming Instructions
Position of vials
Position 1: borate buffer
Position 2: water for needle wash (open vial)
Position 3: OPA reagent
Position 4: water
Position X: sample (amino acids standard, BSA, test article)
X is any other position of the autosampler tray.
Derivatization/injection routine:
1 Draw 2.5 µL from vial 1 (borate buffer)
2 Draw 2.5 µL from sample vial (e.g., position #11)
3 Mix 3 µL in air, max speed, 2X
4 Wait 0.5 min
5 Draw 0 µL from vial 2 (needle wash)
6 Draw 0.5 µL from vial 3 (OPA reagent)
7 Mix 3.5 µL in air, max speed, 6X
8 Draw 0 µL from vial 2 (needle wash)
9 Draw 32 µL from vial 4 (water)
10 Mix 18 µL in air, max speed, 2X
11 Inject

Auxiliary instructions: draw speed, 200 µL/min; eject speed, 600 µL/min; draw position, 0.0 mm.

(Figure 2) assess the completeness of the hydrolysis step. In addition, L-norvaline, which is added as the internal standard, provides a control for sample-to-sample variability.

Specificity

Specificity was documented by comparing retention times obtained in the standard amino acid mixture (five samples) with those obtained from the reference protein samples (three samples). Results are reported in Table 2. The minimal difference between retention times (<0.1%) allows confident, highly specific, peak identification. Usually, a difference within ±3% is considered acceptable between retention times of the same amino acids present in the standard mixture and in the hydrolyzed sample.[5]

Linearity and Range

Linearity was studied in the range from 20 to 500 pmol/µL of standard amino acids and from 1.5 to 15 pmol/µL of rFab, corresponding approximately to 20–200% of the test concentration. Five concentration points were assayed in duplicate. Both the standard amino acid mixture and the test product showed good linearity in the tested range. The area response obeyed the equation $y = mx + C$, where the intercept C was zero within 95% confidence limits and the square correlation coefficient (R^2) was always greater then 0.985 (Table 3).

Accuracy

Accuracy is defined as the agreement between the found value and the true, independently determined, concentra-

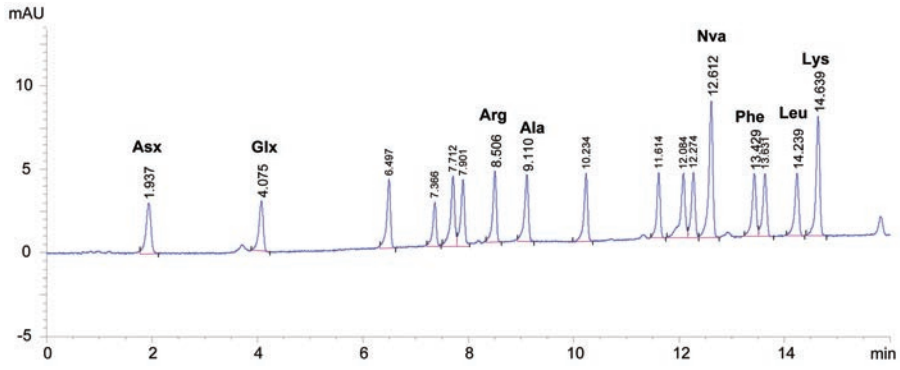

FIGURE 1

Example of a standard amino acid mixture analysis at a concentration of 250 pmol/µL.

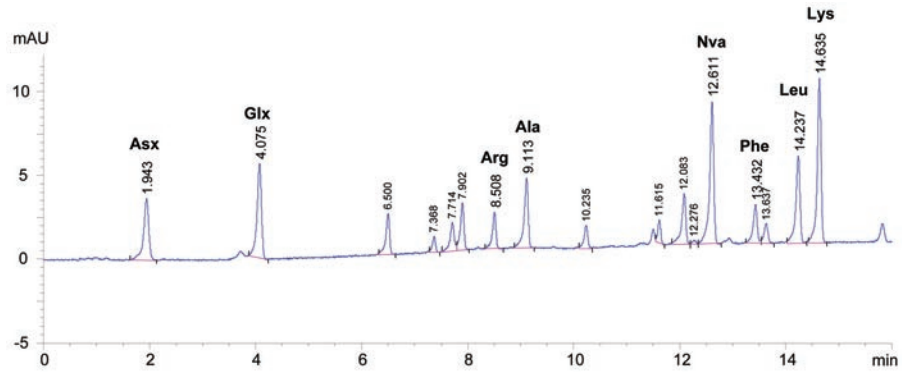

FIGURE 2

Example of a protein hydrolysate analysis for one of the 35-µg BSA samples.

tion value. It was studied on BSA samples prepared from a calibrated standard, which is normally used in protein determination assays. The accuracy of the method was evaluated considering two different parameters: the absolute percent error and the recovery. The absolute percent error (%ABS) was calculated for each well-recovered amino acid. It was always less then 7%, and the average percent error was less then 2.5% (Table 4). Based on the found amino acid concentrations and the known composition of BSA, the percent recovery of BSA was calculated. Considering each single amino acid, the recovery was found within the range 97–108%, while the average recovery, considering all the amino acids, was 102% (Table 5), which is largely within the 90–110% range that is considered acceptable.[5]

Precision

Precision was measured as repeatability and intermediate precision. Reproducibility, which refers to the use of the analytical procedure in different laboratories, was beyond the scope of the present study.

Repeatability was studied on six injections (and derivatization) of the same BSA sample. The mean accuracy of each well-recovered amino acid was calculated. The percentage coefficient of variation (%CV) for each well-recovered amino acid was less than 2.5% (Table 6), compared with an acceptance range of 5%.

Intermediate precision was studied by running the whole method on three different days. Each day, three equivalent BSA samples were prepared, hydrolyzed, derivatized, and injected. The %CV for each well-recovered amino acid was generally not greater than 2.5%, with only one exception for arginine at 8%, as reported in Table 7.

Quantification and Detection Limits

The limit of quantification (LOQ) and the limit of detection (LOD) were calculated on the basis of the standard deviation of the response and the slope obtained from the linearity plot of each well-recovered amino acid of the standard mixture, as described in the relevant ICH guideline.[6] LOQ and LOD were calculated as $3.3\alpha/S$ and $10\alpha/S$, respectively, where α is the standard deviation of the *y*-intercept and S is the slope of regression line. The

TABLE 2

Specificity Evaluation Comparing Retention Times

	AA Std. Sol. (n = 5)		BSA (n = 3)		
	Tr (min)	**SD** (min)	**Tr** (min)	**SD** (min)	Difference (%)
Asx	1.895	0.0018	1.897	0.0020	0.08
Glx	4.019	0.0045	4.018	0.0000	−0.03
Arg	8.469	0.0025	8.467	0.0010	−0.02
Ala	9.030	0.0050	9.028	0.0015	−0.02
Phe	13.305	0.0065	13.304	0.0026	−0.01
Leu	14.099	0.0061	14.097	0.0015	−0.02
Lys	14.494	0.0055	14.492	0.0015	−0.01

TABLE 3

Linearity Data for Amino Acid Standard Mixture and rFab

	Amino acid standard mixture			rFab		
	Slope	Intercept	R^2	Slope	Intercept	R^2
Asx	0.0019	−0.0119	0.9986	0.0112	+ 0.0034	0.9921
Glx	0.0017	−0.0113	0.9984	0.0181	− 0.0261	0.9933
Arg	0.0020	−0.0084	0.9972	0.0277	− 0.0079	0.9856
Ala	0.0021	−0.0132	0 9976	0.0127	− 0.0186	0.9904
Phe	0.0021	−0.0137	0 9982	0.0054	+ 0.0062	0.9900
Leu	0.0020	−0.0158	0 9980	0.0150	− 0.0184	0.9933
Lys	0.0035	−0.0199	0 9956	0.0180	+ 0.0136	0.9980

calculated values of LOQ and LOD for each considered residue are reported in Table 8.

CONCLUSIONS

Method specificity was demonstrated by comparing the retention times for the considered amino acids in hydrolyzed BSA samples and amino acid standard solutions. Given the excellent reproducibility of retention times between amino acid standard solution and protein samples, the method proved highly specific.

Linearity was demonstrated for single amino acid response both in recombinant Fab and in standard amino acid samples in a range of 25–150% of the usually analyzed amount. The square correlation coefficient was above 0.985, with only one case (arginine) below 0.99.

The LOQ and the LOD were in the range 33–60 pmol/μL and 11–20 pmol/μL, respectively, depending on the amino acid under consideration.

Accuracy was determined by comparison of found amino acid residue numbers with those obtained from BSA known composition. The average absolute error was 2.33%. In addition, BSA recovery was determined both from single amino acids (range 97–108%) and as an average value (102%).

Repeatability was measured by carrying out analyses on six injections (including derivatization) of the same hydrolyzed BSA sample. The %CV for each considered amino acid was less than 5%.

Intermediate precision was determined on three different days, by processing three equivalent BSA samples each day. For single amino acid recovery, the %CV for each considered amino acid was less than 8%, with only one case (arginine) above 2.5%.

The determined validation parameters are in the commonly acceptable ranges for this kind of analysis, and allow the use of the method with various recombinant proteins (e.g., antibody fragments) both as an identity test and for extinction coefficient determination when the sequence in known.

An important contribution to these results came from the automation adopted for amino acid derivatization,

TABLE 4

Absolute Percent Error (%ABS) of Found to Theoretical (Nt) Residue Numbers for the BSA Molecule

	Nt	Inj. #1	Inj. #2	Inj. #3	Mean ± SD	%ABS
Asx	54	54.2	53.7	54.7	54 ± 0.45	0.34
Glx	79	82.0	81.7	76.8	80 ± 2.93	1.49
Arg	23	20.9	27.0	25.8	25 ± 3.19	6.99
Ala	47	45.0	44.5	45.0	45 ± 0.29	4.60
Phe	27	26.7	26.9	28.0	27 ± 0.67	0.77
Leu	61	61.0	59.9	61.6	61 ± 0.85	0.30
Lys	59	59.9	56.1	57.9	58 ± 1.93	4.78
Avg. %ABS						2.33

TABLE 5

BSA Recovery, as Calculated from Single Amino Acids

	Inj. #1 (µg)	Inj. #2 (µg)	Inj. #3 (µg)	Mean ± SD (µg)	Recovery %
Asx	34.7	36.1	35.9	35 .6 ± 0.8	102
Glx	35.9	37.5	34.5	36.0 ± 1.5	103
Arg	31.5	42.6	39.7	37.9 ± 5.7	108
Ala	33.1	34.3	34.0	33.8 ± 0.6	97
Phe	34.3	36.1	36.8	35.7 ± 1.3	102
Leu	34.6	35.6	35.8	35.3 ± 0.6	101
Lys	35.1	34.5	34.8	34.8 ± 0.3	99
Average Recovery %					102

TABLE 6

Repeatability (Numbers of Residues)

	Nt	Inj. #1	Inj. #2	Inj. #3	Inj. #4	Inj. #5	Inj. #6	Mean ± SD	%CV
Asx	54	54.9	55.2	53.7	54.6	54.5	53.9	54.5 ± 0.54	1.00
Glx	79	83.6	83.0	82.0	83.9	83.7	83.7	83.3 ± 0.69	0.83
Arg	23	20.7	21.3	21.5	21.6	20.5	21.4	21.2 ± 0.47	2.21
Ala	47	44.8	44.7	46.6	44.7	44.3	44.7	45.0 ± 0.80	1.78
Phe	27	27.2	28.0	27.7	26.7	28.3	27.5	27.6 ± 0.54	1.97
Leu	61	62.3	62.0	61.3	61.2	61.5	61.5	61.6 ± 0.44	0.72
Lys	59	56.2	55.8	57.0	57.1	57.1	57.0	56.7 ± 0.55	0.97

TABLE 7

Intermediate Precision of Well-Recovered Amino Acid Residues (Numbers of Residues)

					Intermediate Precision	
	Nt	Day 1	Day 2	Day 3	Mean ± SD	Percentage
Asx	54	55.2	54.2	55.1	54.8 ± 0.56	101.5 ± 1.03
Glx	79	84.2	80.2	81.7	82.1 ± 2.05	103.9 ± 2.50
Arg	23	21.1	24.6	23.9	23.2 ± 1.85	100.1 ± 7.97
Ala	47	44.4	44.8	45.5	44.9 ± 0.53	95.5 ± 1.17
Phe	27	26.2	27.2	27.3	26.9 ± 0.62	99.6 ± 2.30
Leu	61	60.3	60.8	60.3	60.5 ± 0.30	99.2 ± 0.49
Lys	59	58.5	57.9	55.9	57.4 ± 1.39	97.3 ± 2.42

TABLE 8

LOQ and LOD Values (pmol/µL)

	Asx	Glx	Arg	Ala	Phe	Leu	Lys
LOQ	34	37	48	44	39	41	61
LOD	11	12	16	15	13	14	20

which guarantees highly reproducible reaction times and lack of degradation. In addition, the short reaction times seem not to give rise to multiple derivatives, as previously observed.[2] Also the use of absorbance instead of fluorescence detection did not negatively influence the performance characteristics of the method.

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