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APPLICATION OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING PRE-COLUMN DERIVATIZATION WITH *o*-PHTHALDIALDEHYDE FOR THE QUANTITATIVE ANALYSIS OF AMINO ACIDS IN ADULT AND FETAL SHEEP PLASMA, ANIMAL FEEDS AND TISSUES

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SUMMARY

The concentration of amino acids in adult and fetal sheep plasma, liver, muscle and animal feeds was determined by reversed-phase high-performance liquid chromatography using an automated data acquisition system and pre-column *o*-phthaldialdehyde derivatization. Of the seventeen amino acids in the standard mixture, fifteen including glycine and threonine were completely resolved in a total analysis time of 50 min. The clear resolution, high degree of precision and accuracy, relatively rapid analysis and the lack of interference from chemical contaminants in feed and tissue hydrolysates render this technique suitable for routine analysis of large numbers of biological samples of nutritional interest.

INTRODUCTION

The use of high-performance liquid chromatography (HPLC) for the quantitative determination of amino acids in biological samples has received wide popularity in recent years due to its sensitivity and speed of analysis. The formation of dansyl[1,2], dabsyl[3,4], and phenylthiohydantoin[5,6] derivatives has rendered the technique extremely sensitive which is particularly desirable under constraints of low concentration or small sample size. However, these derivatives were found to lack sufficient specificity for amino acid analysis in complex biological materials. Following the report by Roth[7] that primary amines yield strongly fluorescent derivatives with *o*-phthaldialdehyde (OPA) in the presence of 2-mercaptoethanol or ethanethiol, several workers have employed pre-column[8–18] or post-column[19] OPA derivatization procedures for amino acid analysis by HPLC. The basis of fluorometric amino acid analysis with OPA and its applications have been reviewed[20]. Concomitant advances in high-pressure delivery pumps capable of microflow rates, microprocessor controlled solvent gradients, better column designs and superior stationary phases have further contributed to the high versatility of this technique[21]. The analytical aspects of HPLC of amino acids, particularly the effect of eluting solvent gradients, nature

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of the stationary phase and the relative merits of pre-column and post-column derivatization have been discussed[22].

The need for a rapid, sensitive and reliable method of amino acid determination as an alternative to the laborious and time consuming ion-exchange chromatography has long been felt. This is especially true in nutritional and metabolic studies which involve the routine analysis of large numbers of plant materials and biological samples. During the course of investigations on protein synthesis in the pregnant ewe and the fetus, we found that relatively little information was available on the application of HPLC for amino acid analysis in fetal plasma, animal feeds and tissues. The present study was therefore undertaken to standardize semi-automated quantitative procedures for the determination of amino acids in these materials using OPA pre-column derivatization and fluorescence detection.

EXPERIMENTAL

Reagents

HPLC-grade methanol and acetonitrile were obtained from Fisher Scientific. Water used for the preparation of buffers and dilutions of standards was distilled in glass and demineralized using a standard Barnstead resin bed (Fisher). The NaH_2PO_4, Na_2HPO_4, H_3BO_3 and KOH were all Fisher certified ACS-grade. The *o*-phthaldialdehyde and the amino acid standard mixture (AA-S18) were purchased from Sigma. The ethanethiol (ETSH) was a product of Eastman-Kodak.

Methods

Solvent preparation. A stock buffer of 300 m*M* phosphate–518 m*M* sodium was prepared by dissolving 11.6 g $NaH_2PO_4 \cdot H_2O$ and 30.7 g Na_2HPO_4 in 1 l of water and adjusting the pH to 7.2 using concentrated hydrochloric acid. Solvent A was prepared as needed by diluting 25 ml of stock buffer with 475 ml of water to yield a final concentration of 15 m*M* phosphate–25.9 m*M* sodium, and subsequently filtered and degassed by vacuum through a 0.45-μm membrane filter (Millipore). Solvent B was prepared by diluting 25 ml of stock buffer in 219 ml of water and 256 ml of acetonitrile, followed by filtration through a 0.5-μm membrane filter.

Borate buffer. A 0.5 *M* borate buffer was made by dissolving 31 g boric acid in 1 l of water and titrating to pH 10.5 with potassium hydroxide pellets.

Amino acid standard. The amino acid standard was prepared by diluting the commercial preparation containing 17 L-amino acids (2.5 μM) to a concentration of 0.5 μM with 0.1 *M* hydrochloric acid and stored at 4°C for a maximum of two weeks. Ethanolamine, asparagine, glutamine, citrulline, tryptophan, 1-methylhistidine and 3-methylhistidine (Sigma) were purchased separately.

Derivatizing reagents. The OPA reagent was made by dissolving 20 mg OPA in 2 ml of methanol in an 8-ml glass screw-capped, foil-covered vial. The ETSH reagent was prepared by diluting 50 μl of ETSH in 5 ml of methanol in a similar vial. Both derivatizing reagents were prepared fresh daily.

Preparation of biological samples for derivatization. Whole blood was obtained from the jugular vein of pregnant ewes and the inferior vena cava of their fetuses at approximately 120 days of gestation through previously implanted vascular catheters[23]. Pieces of the caudate lobe of the liver and the gastrocnemius muscle were

excised from the ewe following sacrifice and immediately dropped in liquid nitrogen. The samples were stored at −20°C until they were analyzed. Alfalfa cubes (Tilley, Canada) and barley (East Chilliwack, Surrey, Canada) used for analysis were sampled at weekly intervals from the feed given to these ewes.

Plasma was prepared by loading 0.2 ml aliquots onto an activated Sep-Pak C_{18} cartridge (Waters Assoc.) and eluting the amino acids first with 1 ml of water and then with 1 ml of 30% methanol. Feed and tissue hydrolysates were prepared by accurately weighing 0.1 g of sample (alfalfa, barley, muscle or liver) into clean 100 × 13 mm screw-capped test tubes and adding 1 ml of 6 *M* hydrochloric acid (2 ml for feeds). The tubes were capped and hydrolyzed overnight (20 h) at 110°C after which the caps were removed and the hydrochloric acid was evaporated from the samples under vacuum. These samples were then reconstituted in 2 ml of 0.1 *M* hydrochloric acid and applied to the Sep-Pak cartridges. The amino acids were eluted using two 1-ml aliquots of water and then two 1-ml aliquots of 30% methanol. The prepared hydrolysates were further diluted as required in 0.1 *M* hydrochloric acid (1:5) to bring them within the range of the calibration curve.

Derivatization procedure. The OPA/ETSH amino acid derivatives were prepared basically by the procedure of Hill *et al.*[8] with modifications. To 200 μl of amino acid standard or sample in 8-ml glass screw-capped, foil-covered vials were added 50 μl of borate buffer, 50 μl of methanol, 100 μl of OPA reagent, and 100 μl of ETSH reagent. The vials were capped, shaken, and allowed to react for 5 min, and then 5 ml of methanol were added. Injections of 10 μl were made into the HPLC system immediately after derivatization.

Chromatographic system

The HPLC system used (Waters Assoc.) consisted of Model 680 automated gradient controller, Model 510 HPLC pumps, Model U6K injector, and Model 420 fluorescence detector. The fluorometer was set at an excitation wavelength of 338 nm and emission cut-off at 425 nm.

A μBondapak C_{18} reversed-phase column (10-μm particle size, 300 × 3.9 mm I.D.) was used in conjunction with a stainless-steel guard column (20 × 3.9 mm I.D.) which was packed with Waters' Corasil C_{18} packing material. The temperature of the column was maintained at 28°C by encasing it in an acrylic jacket connected to a Braun circulating water bath by polyethylene tubing.

The data collecion system consisted of an Apple IIe computer, a two channel ISAAC interface and a 42A CHROMATEXT software package (Cyborg Corporation, Newton, MA, U.S.A.). In addition a strip chart recorder was used for real-time monitoring of peak elution.

HPLC conditions. The flow-rate was maintained at 2 ml/min and a linear gradient (Curve 6) changing from 75% to 25% Solvent A over a time period of 50 min. A 3-min linear gradient at the end of each run brought the system back to initial conditions; *i.e.*, A–B (75:25).

Quantitative analysis

Peaks were identified with reference to the retention times of standard amino acids injected separately. The peak areas of known concentrations of authentic amino acids were measured using the software (CHROMATEXT) supplied by Cyborg Cor-

poration. Ethanolamine was used as the internal standard to account for injection variations. The linearity of response was estimated by injecting different concentrations of derivatized amino acids and constructing regression equations of fluorescence response–concentration curves. The within-run precision at different concentrations was estimated by injecting different volumes of derivatized standards in replicates and the between-run precision by analyzing aliquots of the same standard on different days. The accuracy of measurement was tested by adding known quantities of amino acids to the plasma and calculating the percentage of recovery. The detection limit was determined by injecting OPA amino acid derivatives from serially diluted standards until the peaks were indistinguishable from the base line.

RESULTS AND DISCUSSION

An elution profile of the amino acid in the standard mixture is given in Fig. 1. In general, the elution order and relative retention times are comparable to those reported by other workers[8,11,12,15,16] who have used similar chromatographic equipment and pre-column OPA derivatization. The notable differences from the observations of Hill *et al.*[8] were the elution of histidine prior to serine and that of lysine before valine under the conditions of this study. As shown by Lindroth and Mopper[9] the elution order of amino acids will be determined by complex interactions between specific ions in the eluent and the OPA derivatives. Of the seventeen L-amino acids and ammonia in the standard mixture, fifteen were clearly resolved. The linear gradient used was particularly advantageous in the resolution of glycine and threonine,

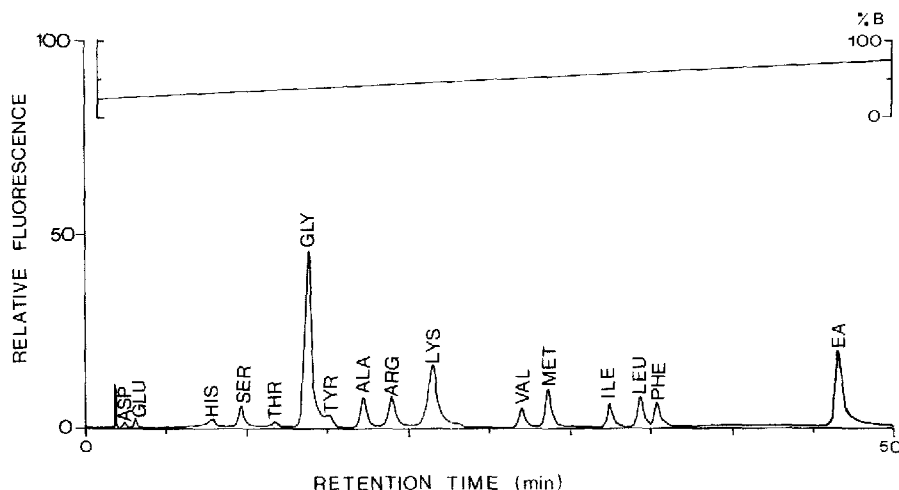

Fig. 1. Chromatogram of standard OPA amino acid derivatives (364 pmol each) on μBondapak C_{18} reversed-phase column (10-μm particle size, 300 × 3.9 mm I.D.). Operating conditions: column temperature 28°C; gradient elution, mobile phase: A = 15 m*M* sodium phosphate buffer, pH 7.2; B = 15 m*M* sodium phosphate buffer, pH 7.2-acetonitrile (1:1); linear gradient from 75% (initial) to 25% (final) solvent A in 50 min at a flow-rate of 2 ml/min. Peaks: ASP = aspartate; GLU = glutamate; HIS = histidine; SER = serine; THR = threonine; GLY = glycine; TYE = tyrosine; ALA = alanine; ARG = arginine; LYS = lysine; VAL = valine; MET = methionine; ILE = isoleucine; LEU = leucine; PHE = phenylalanine; EA = ethanolamine (internal standard).

TABLE I

EFFECT OF TEMPERATURE ON THE RELATIVE RETENTION TIMES (t_R) OF PRE-COLUMN OPA-AMINO ACID DERIVATIVES

Amino acid	*t_R at different column temperatures*					
	22–24°C			*28°C**		
	*Mean***	*S.D.*	*C.V. (%)*	*Mean****	*S.D.*	*C.V.(%)*
ASP	0.070	0.006	9.2	0.062	0.001	2.2
GLU	0.103	0.007	6.3	0.080	0.002	2.9
HIS	0.251	0.004	1.4	0.209	0.011	5.4
SER	0.328	0.002	0.6	0.255	0.015	5.9
THR	0.371	0.004	1.2	0.313	0.017	5.3
GLY	0.430	0.001	0.3	0.367	0.018	4.9
TYR	0.445	0.003	0.7	0.432	0.009	2.0
ALA	0.531	0.004	0.8	0.484	0.023	4.7
ARG	0.596	0.005	0.8	0.537	0.011	2.1
LYS	0.632	0.004	0.7	0.601	0.008	1.4
VAL	0.786	0.006	0.7	0.742	0.028	3.7
MET	0.827	0.005	0.6	0.799	0.009	1.1
ILE	0.921	0.003	0.4	0.912	0.004	0.4
LEU	0.968	0.003	0.3	0.970	0.000	0.0
PHE§	1.000	0.000	0.0	1.000	0.000	0.0
EA§§	1.402	0.006	0.4	1.359	0.010	0.7

* All the t_R values at 28°C were lower than at 22–24°C ($P < 0.05$).
** Calculated from three measurements during 1 week at 22–24°C.
*** Calculated from 26 measurements over a 2-month period at 28°C.
§ Phenylalanine taken as unity.
§§ Ethanolamine used as an internal standard.

the separation of which required the use of different buffer systems[11,14,15,18,24] or a 3-μm particle-size column[16].

Initially the system was run at room temperature (22–24°C). Under this condition tryptophan co-eluted with leucine. To overcome this problem, the modification suggested by Hill *et al.*[8] was followed by maintaining the column at 28°C, which facilitated the separation of these two amino acids without losing resolution of others. Increasing the column temperature from 22 to 28°C significantly ($P < 0.05$) decreased the retention times of all amino acids (Table I) which confirms the findings of Hill *et al.*[15]. The overall coefficient of variation (C.V.) of the retention times at 28°C over a 2 month period was less than 6% in all cases indicating the stability of the column.

Cystine was not resolved because it has to be converted to a derivative of iodoacetic acid in order to form a highly fluorescent adduct[20]. Secondary amino acids such as proline do not form OPA derivatives readily[7] and this peak was not detected at the concentration used. The ammonia peak was also not detected. These observations confirm earlier reports[12,18]. Citrulline, asparagine, and 3-methylhistidine were incompletely resolved from glycine, histidine, and tyrosine respectively. The retention times of glutamine, 1-methylhistidine and tryptophan relative to phenylalanine were 0.27, 0.41 and 0.94, respectively.

It takes 50 min for a complete run and an additional period of 3 min for the

TABLE II

MOLAR RESPONSE OF FLUORESCENT OPA AMINO ACID DERIVATIVES

Amino acid	*Response (pmol)*	*Response relative to glycine*	*Rank**
ASP	3.883	0.021	15
GLU	11.859	0.065	14
HIS	41.461	0.228	6
SER	38.128	0.210	9
THR	13.047	0.072	13
GLY	181.976	1.000	1
TYR	42.005	0.231	5
ALA	42.231	0.232	4
ARG	28.546	0.157	10
LYS	71.795	0.395	2
VAL	27.259	0.150	12
MET	49.354	0.271	3
ILE	39.723	0.218	8
LEU	40.487	0.223	7
PHE	27.562	0.152	11

* Ranking from highest intensity (1) to lowest intensity (15).

TABLE III

THE FLUORESCENT RESPONSE OF OPA AMINO ACID DERIVATIVES AND PRECISION OF DETERMINATION

Amino acid	*Fluorescent response*		*Standard error of the estimate*	*Precision*	
	*Regression equation**	*Coefficient of determination (r^2)*		*C.V. (%) within run***	*C.V. (%) between run****
ASP	$y = 106.7x - 132.6$	0.967	0.155	3.0	6.1
GLU	$y = 184.8x + 101.4$	0.992	0.076	4.5	6.6
HIS	$y = 664.9x + 895.8$	0.977	0.068	6.3	5.6
SER	$y = 569.8x + 274.4$	0.983	0.114	2.4	4.4
THR	$y = 383.5x + 550.2$	0.959	0.164	6.3	5.3
GLY	$y = 3332.0x + 47.0$	0.999	0.049	3.3	7.0
TYR	$y = 734.1x - 193.2$	0.995	0.083	8.4	5.7
ALA	$y = 601.8x + 792.6$	0.976	0.060	2.2	3.6
ARG	$y = 533.3x - 86.4$	0.999	0.030	4.0	3.9
LYS	$y = 1545.2x - 784.8$	0.993	0.117	1.8	6.8
VAL	$y = 499.2x + 11.4$	0.999	0.060	3.6	5.4
MET	$y = 946.8x - 334.8$	0.999	0.032	1.5	6.1
ILE	$y = 478.2x + 678.4$	0.921	0.202	7.7	5.0
LEU	$y = 608.2x + 530.6$	0.987	0.061	2.3	3.6
PHE	$y = 638.1x - 514.0$	0.987	0.084	3.2	4.3

* Regression equation is in the form $y = mx + b$, where y is the peak area, m is the slope, x is the amino acid concentration and b is the y intercept.

** Within-run coefficient of variation is based on three injections of the standard mixture of amino acids on the same day.

*** Between-run coefficient of variation is based on the mean coefficients of variation of six days over a two month period with $n = 14$.

system to return to the initial condition. Though this time is much shorter than that required for conventional ion-exchange chromatography, it has been claimed that pre-column derivatization with phenyl isothiocyanate (Pico-Tag, Waters Assoc.) facilitates a single run to be completed within 15 min.

The molar response of the fluorescent OPA amino acid derivatives relative to glycine is given in Table II. The differential stability of the fluorescence of various OPA amino acid derivatives has been reported by many workers[8,11,18,25,26]. To minimize fluctuations in fluorescence response due to these variations it has been suggested[13] that the time between derivatization and injection be kept constant. Accordingly, the technique of injecting the samples immediately after derivatization was adopted. The fluorescence intensities of glycine and lysine OPA derivatives were relatively high whereas those of aspartate, glutamate and threonine were low. Other amino acid derivatives occupied an intermediate position. The fluorescence response of the lysine OPA derivative in this study is contrary to the report of Hogan *et al.*[14] who observed a low response. The differences in the derivatization conditions and the excitation wavelengths used in the fluorometer in the two studies may explain the discrepancy. The detection limit for glycine and lysine was 9 pmol while for others it was 18 pmol.

Though many data acquisition systems have been developed recently to collect, store and retrieve liquid chromatographic data, they are designed in such a way that they can only be used in conjunction with the chromatographic equipment. In order to enhance the potential use of the data acquisition system to other commonly used analytical and physiological recording devices in the laboratory, a more versatile system would be preferable. The hardware and software used in this study permit the identification of peaks based on retention times, correction for background noise and elimination of low peaks as specified. The peak area and/or the peak height can be measured manually or automatically by programming the system to integrate the areas and store the data for retrieval at a later date. The system also permits the

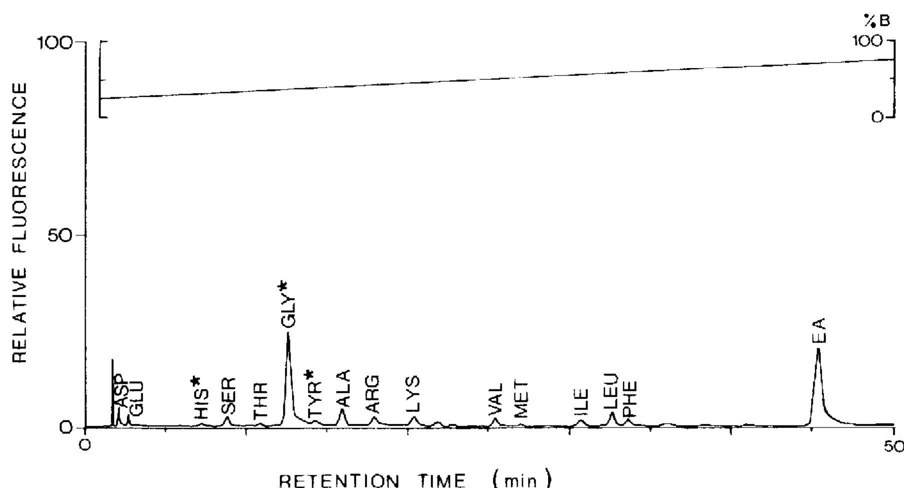

Fig. 2. Chromatogram of OPA-amino acids in alfalfa cube hydrolysate. Conditions are the same as in Fig. 1. (★) Histidine, glycine and tyrosine peaks include asparagine, citrulline and 3-methylhistidine respectively. Unmarked peaks were not identified.

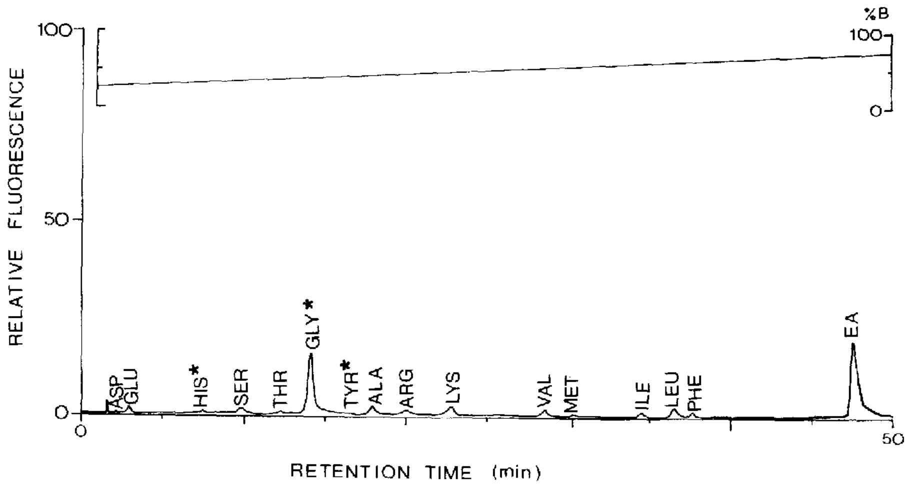

Fig. 3. Chromatogram of OPA-amino acids in barley hydrolysate. Conditions are the same as in Fig. 1. (★) Histidine, glycine and tyrosine peaks include asparagine, citrulline and 3-methylhistidine respectively. Unmarked peaks were not identified.

comparison of different chromatographic runs with one another or with standards. For the quantitation of amino acids, peak areas were measured and regression equations developed from the fluorescence response—concentration curve of each amino acid (Table III).

The application of HPLC has been found to be particularly advantageous for amino acid analysis in fetal plasma where sample size is usually limiting and in animal feeds and tissues where the speed of analysis is as important as analytical efficiency. Representative chromatograms of amino acids in the hydrolysates of alfalfa cubes,

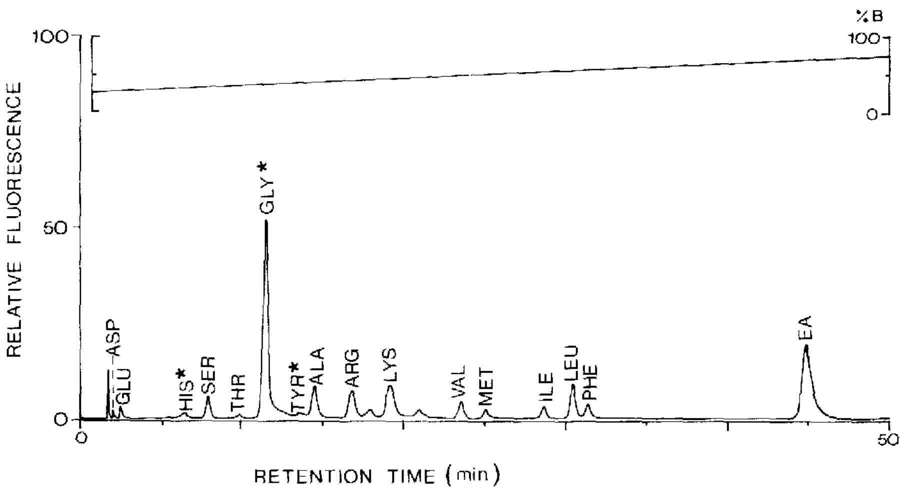

Fig. 4. Chromatogram of OPA-amino acids in sheep liver hydrolysate. Conditions are the same as in Fig. 1. (★) Histidine, glycine and tyrosine peaks include asparagine, citrulline and 3-methylhistidine respectively. Unmarked peaks were not identified.

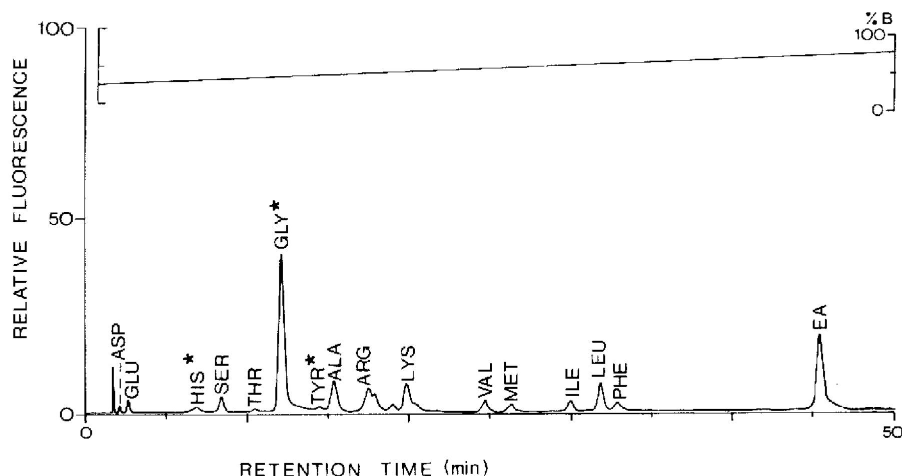

Fig. 5. Chromatogram of OPA-amino acids in sheep gastrocnemius muscle. Conditions are the same as in Fig. 1. (★) Histidine, glycine and tyrosine peaks include asparagine, citrulline and 3-methylhistidine respectively. Unmarked peaks were not identified.

barley, ovine liver and muscle are shown in Figs. 2–5. The recovery of amino acids added to the plasma was 98.3 ± 5.2% (Table IV) showing the level of accuracy of the method. The clear resolution of peaks without baseline drifts or interference from organic and inorganic compounds usually present in feeds and tissues demonstrates the usefulness of the HPLC procedure for amino acid analysis in these materials as well as the efficiency of sample clean-up with the Sep-Pak cartridges. However, under the conditions of this study, it was not possible to resolve histidine and asparagine,

TABLE IV

RECOVERY OF AMINO ACID STANDARD ADDED TO PLASMA

Amino acid	*Amino acid per injection (pmol)*			
	Plasma alone	*Plasma + 36.36 pmol (actual)*	*Plasma + 36.36 pmol (predicted)*	*Recovery (%)*
ASP	8.80	47.14	45.16	104.38
GLU	18.58	56.01	54.94	101.95
HIS	19.65	57.14	53.03	107.75
SER	3.02	40.94	39.38	103.96
THR	32.83	69.85	69.19	100.95
GLY	32.83	69.85	69.19	100.95
TYR	64.99	100.57	101.35	99.23
ALA	64.99	100.57	101.35	99.23
ARG	16.16	49.60	52.52	94.44
LYS	75.28	111.26	111.64	99.66
VAL	25.02	56.14	61.38	91.46
MET	5.05	38.23	41.41	92.32
ILE	14.51	47.25	50.87	92.88
LEU	64.18	91.46	100.53	90.98
PHE	5.93	39.98	42.29	94.54

TABLE V

AMINO ACID CONCENTRATION IN BIOLOGICAL SAMPLES

Amino acid	*Percentage of amino acid in sample**					
	*Alfalfa cubes*** *(n = 4)*	*Barley**** *(n = 4)*	*Muscle*§ *(n = 4)*	*Liver*§ *(n = 4)*	*Maternal plasma*§§,§§§ *(n = 11)*	*Fetal plasma*§§,§§§ *(n = 15)*
ASP	1.52 ± 0.22	0.99 ± 0.06	1.54 ± 0.13	1.50 ± 0.25	0.25 ± 0.04	0.25 ± 0.02
GLU	0.78 ± 0.12	1.17 ± 0.14	2.29 ± 0.21	1.55 ± 0.10	1.40 ± 0.14	1.02 ± 0.13
HIS	0.06 ± 0.01	0.05 ± 0.00	0.03 ± 0.00	0.30 ± 0.02	0.23 ± 0.04	0.25 ± 0.02
SER	0.57 ± 0.06	0.56 ± 0.02	0.72 ± 0.04	1.25 ± 0.04	0.16 ± 0.03	0.83 ± 0.13
THR		1.04 ± 0.11	0.55 ± 0.06	0.63 ± 0.06	0.40 ± 0.09	0.51 ± 0.15
GLY	0.81 ± 0.01	0.65 ± 0.06	1.20 ± 0.07	1.81 ± 0.13	0.17 ± 0.02	0.21 ± 0.02
TYR	0.25 ± 0.04		0.35 ± 0.03	0.41 ± 0.02	0.03 ± 0.00	0.10 ± 0.01
ALA	0.64 ± 0.02	0.52 ± 0.05	1.35 ± 0.06	2.04 ± 0.05	0.12 ± 0.01	0.17 ± 0.02
ARG	1.41 ± 0.20	0.88 ± 0.07	5.44 ± 0.36	4.42 ± 0.14	0.08 ± 0.01	0.11 ± 0.01
LYS	0.47 ± 0.05	0.68 ± 0.05	1.30 ± 0.06	2.04 ± 0.08	0.12 ± 0.03	0.13 ± 0.03
VAL	0.40 ± 0.03	0.76 ± 0.10	1.17 ± 0.03	0.66 ± 0.05	0.36 ± 0.04	0.40 ± 0.06
MET	0.57 ± 0.06	0.28 ± 0.00	0.56 ± 0.03	1.48 ± 0.05	0.03 ± 0.00	0.04 ± 0.01
ILE	0.45 ± 0.04	0.39 ± 0.05	1.04 ± 0.03	1.50 ± 0.12	0.18 ± 0.02	0.10 ± 0.01
LEU	0.89 ± 0.05	0.86 ± 0.08	2.34 ± 0.07	3.54 ± 0.40	0.20 ± 0.02	0.13 ± 0.02
PHE	0.70 ± 0.06	0.78 ± 0.04	0.96 ± 0.06	1.49 ± 0.10	0.07 ± 0.01	0.08 ± 0.01

* Values represent mean ± standard deviation of *n* replicates.
** Alfalfa hydrolysate prepared from dried, ground alfalfa cubes.
*** Barley hydrolysate prepared from dried, ground whole barley.
§ Liver and muscle hydrolysates prepared from frozen, thawed, wet tissue samples.
§§ Based on peak height regression data.
§§§ Plasma values expressed in μmol/ml of plasma ± standard error.

glycine and citrulline, and tyrosine and 3-methylhistidine and, if these compounds are of interest, changes have to be made in the chromatographic conditions to separate them. The involvement of citrulline as a component of the urea cycle in the liver, the delivery of a substantial amount of asparagine to the sheep fetus, and the use of 3-methylhistidine for the quantitation of muscle protein degradation would be specific instances in which complete resolution of these amino acids will be necessary. The amino acid concentrations in the biological samples analyzed are given in Table V.

The absence of a tryptophan peak in feed and tissue hydrolysates and its presence in the unhydrolyzed plasma support the contention[27] that tryptophan undergoes degradation during the course of acid digestion of biological samples.

It may be concluded that the clear resolution of amino acids, the high degree of precision and accuracy, the relatively rapid analysis requiring less than 53 min per sample including the time required to return to the initial conditions, and the lack of interference from chemical contaminants in the hydrolysates, render the reversed-phase HPLC technique using precolumn derivatization with OPA suitable for the routine analysis of large numbers of biological samples in nutritional investigations.

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