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Isocratic high-performance liquid chromatographic determination of tryptophan in infant formulas☆

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Abstract

The application to infant formulas of a method for tryptophan determination by isocratic HPLC with UV detection at 254 nm, after derivatization with phenyl isothiocyanate, was studied. Protein was hydrolysed by barium hydroxide at 120°C for 8 h, followed by derivatization with phenyl isothiocyanate, HPLC and UV detection at 254 nm. The optimum chromatographic conditions (pH, ionic strength of elution solvent and eluent ratio) were established. The analytical parameters (linearity, precision, accuracy of derivatization and limits of detection and quantification) were determined. The values obtained demonstrated that the method is useful for determining the tryptophan content of infant formulas. The tryptophan contents in six different types of infant formulas were 0.535–0.848 g per 100 g of protein.

1. Introduction

Tryptophan is an essential amino acid for infants, because it cannot be formed endogenously and must be obtained from infant formulas or human milk. As human milk and infant formulas are the first and only source of nourishment for neonates, the composition must provide the optimum amino acid content in order to meet infant requirements (17 mg/kg daily for infants at 3–4 months old [1]). Since human milk has traditionally been the first and sole food of neonates, it is reasonable to assume that its composition is ideal for meeting newborn infants' requirements for rapid growth, development and maturation [2].

Several methods for tryptophan determination have been developed for pure proteins and peptides involving ion-exchange chromatography [3] or reversed-phase high-performance liquid chromatography (HPLC) with fluorimetric [4,5] or UV–visible detection after chemical derivatization [6–12]. Before the determination of the tryptophan content of proteins, an alkaline hydrolysis [3,13,14] or enzymatic digestion must be carried out [15]. The most effective alkalis used for hydrolysis are LiOH [3,5], NaOH [5,16,17] and $Ba(OH)_2$ [3,12,18]. Hydrolysis for 4–8 h is needed, depending on the type of protein and the hydrolysis temperature, which varies from 100 to 145°C.

In this work, the application to infant formulas of a method for tryptophan determination by HPLC with UV detection at 254 nm, after derivatization with phenyl isothiocyanate (PITC), was studied. After establishing the

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optimum chromatographic conditions (mobile phase, pH, sodium acetate concentration), the analytical parameters of the method were determined and the method was applied to the determination of the tryptophan content in infant formulas.

2. Experimental

2.1. Apparatus

The chromatographic system (Shimadzu, Kyoto, Japan) consisted of two LC-6A pumps controlled by an SCL-6A liquid chromatograph system controller, a Model 7125 manual injection valve (Rheodyne, Cotati, CA, USA) equipped with a 20-μl sample loop and an RF-S3 UV–visible detector (absorbance wavelength 254 nm). Data were collected and analysed with a G-R4A data processor.

Solvents were filtered by using a Millipore (Milford, MA, USA) system with 0.22-μm membrane filters (47 mm) and samples were filtered using a Millipore system with 0.22-μm membrane filters (13 mm).

A UT 6060 air-circulation drying oven (Heraeus, Hanau, Germany) was used in the hydrolysis step and a Telstar (Terrassa, Spain) CO vacuum oil pump, 33 hp (ca. 24 kW), flow-rate 5 m^3/h, was used to remove liquids.

2.2. Reagents

PITC was obtained from Fluka (Buchs, Switzerland), triethylamine (TEA) of analytical-reagent grade from Merck (Darmstadt, Germany), barium hydroxide octahydrate of analytical-reagent grade from Panreac (Barcelona, Spain), L-tryptophan and L-norleucine standard solutions from Sigma (St. Louis, MO, USA) and acetonitrile and ethanol of HPLC grade from Scharlau (Barcelona, Spain). Buffers were prepared with reagents of analytical-reagent grade and all aqueous solutions were prepared with high-purity water produced with a Millipore Milli-Q system.

2.3. Hydrolysis procedure

Samples (500 mg) were weighed in Pyrex glass tubes with Teflon-lined screw-caps used as the hydrolysis vessel. A 4.83-g amount of barium hydroxide and 5 ml of boiling water were then added to the glass tubes and the contents were mixed. Air was removed with nitrogen in an ultrasonic bath for 30 s.

Subsequently the samples were hydrolysed by heating at 120°C for 8 h and, after adjusting the pH to 3–4 with hydrochloric acid, they were filtered through a membrane filter and diluted to 25 ml with water.

2.4. Derivatization procedure

An aliquot of hydrolysate (163 μl) was transferred into Pyrex tubes (60 × 50 mm), then placed in a flask that can hold twelve tubes, which was connected to a vacuum system in order to evaporate the liquid. The internal standard (norleucine) was then added (10 μl of a 4-nmol/μl solution) and vacuum dried.

A volume of ethanol–water–TEA (2:2:1), at the rate of 2 μl of reagent per 100 nmol of amino acid, was added to the samples and standards and then dried under vacuum. The derivatizing solution [ethanol–TEA–water–PITC (7:1:1:1)] was then added to the standards and samples at the rate of 5 μl of solution per 100 nmol amino acid. The derivatizing solution was freshly prepared before use. The tubes with sample and standards were capped and allowed to stand for 20 min at room temperature. The excess of reagents was carefully removed at under vacuum (the vacuum is a very important parameter in order to obtain reproducible data). The derivatized samples can be stored in a freezer (−20°C) for several weeks.

Just before the analysis for tryptophan, the residue was dissolved by adding 400 μl of 5 m*M* sodium acetate buffer (pH 7.4) containing 5% of acetonitrile. The mixture was allowed to stand for 1 min in a sonication bath, then filtered through a 0.22-μm membrane filter.

2.5. Chromatographic procedure

The chromatographic separation was performed on an ODS-2 Spherisorb column (25 cm × 4.6 mm I.D.; 5 μm) at room temperature. Eluent A (pump A) was 17 m*M* sodium acetate in water containing 325 μl/l of TEA (the pH was adjusted to 6.8 with glacial acetic acid) and eluent B (pump B) was acetonitrile. The separation of tryptophan was carried out using isocratic elution with A–B (84:16).

Norleucine was used as the internal standard because it is not a component of milk protein, it is stable in basic media, it reacts quantitatively with PITC and its retention time is very different from that of tryptophan.

2.6. Samples

The method described here was used to determine tryptophan in different types of milk formulas: milk-based ($n = 2$), soy protein based ($n = 1$), without lactose ($n = 1$), hypoallergenic ($n = 1$) and low-birth-mass ($n = 1$).

3. Results and discussion

3.1. Choice of elution conditions: pH and sodium acetate concentration

These tests were designed to find the optimum eluent composition, pH and sodium acetate concentration for tryptophan determination by HPLC. As a starting point, the eluent composition used by other workers was considered. Eluent C was 25 m*M* sodium acetate in water containing 0.5 ml of TEA and eluent D was 50 m*M* sodium acetate–acetonitrile (50:50). The pH of both eluents was adjusted to 6.8 with glacial acetic acid. Good resolution was obtained between tryptophan and the internal standard with isocratic elution with C–D (65:35).

In order to reduce the washing step and since the elution conditions were isocratic, another eluent containing the same amount of sodium acetate and TEA, which is described above (see chromatographic procedure), was prepared. The separation of tryptophan was carried out using isocratic elution with A–B (84:16). Different ratios of this eluent (85:15, 84:16 and 83:17) were tested. The best resolution and the shortest analysis time, especially in samples, were obtained with the A–B (84:16).

The different pH values and three different sodium acetate concentrations were tested. The results obtained are given in Table 1. pH 6.8 was chosen because at pH 6 the analysis time was longer.

The lowest sodium acetate concentration (17 m*M*) was selected because the results were similar with other concentrations and it was less damaging to the column. The analysis time was 25 min.

Typical HPLC traces for a standard and sample (infant formula) obtained by applying the described method are shown in Figs. 1 and 2, respectively.

3.2. Analytical parameters

Limits of detection (LD) and quantification (LQ)

The limits of detection and quantification were determined by the method described by Knoll

Table 1
Eluent conditions

Conditions	Retention time (min)		Relative retention time
	Tryptophan	Norleucine	
Eluent ratio, A:B			
85:15	34.600	17.367	1.99
84:16	27.315	14.438	1.89
83:17	19.350	10.603	1.82
pH			
6	46.260	22.419	2.06
6.8	27.315	14.438	1.89
Sodium acetate concentration (mM)			
34	27.315	14.438	1.89
17	25.769	12.592	2.04

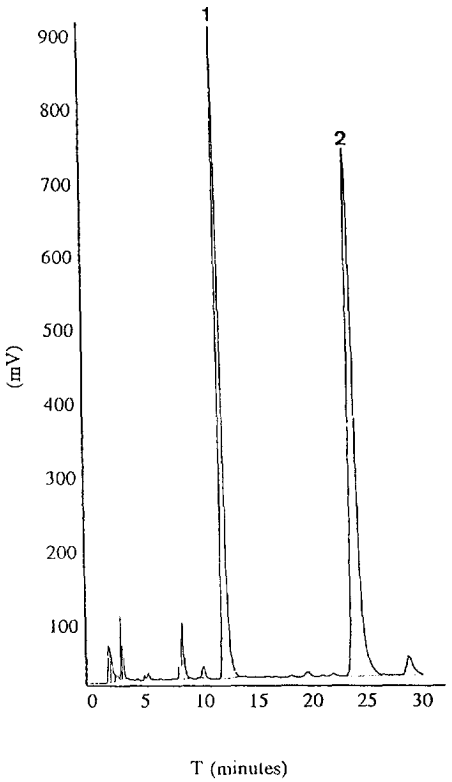

Fig. 1. Chromatogram of standard amino acids (0.1 nmol/μl). Peaks: 1 = norleucine; 2 = tryptophan.

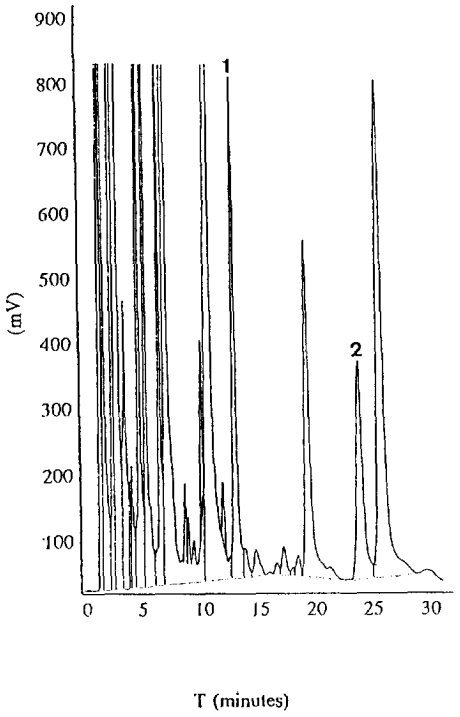

Fig. 2. Chromatogram of infant formula. Peaks: 1 = norleucine; 2 = tryptophan.

[19], the results obtained being 3.9 nmol (18 mg per gram of sample) and 11.3 nmol (51 mg per 100 g of sample), respectively.

Successive dilutions of the lowest standard were injected to ascertain which concentration, following a linear response, had an area ten times the baseline oscillation. The values obtained were LD = 5 nmol and LQ = 10 nmol.

Precision, accuracy of derivatization and linearity

The values obtained in the determination of the relative standard deviations, peak-area ratio and relative retention time precision are given in Table 2. Data, regarding accuracy of derivatization, are summarized in Table 3. The linearity was tested by the analysis of standards containing 20, 40, 100 and 200 nmol of tryptophan. A linear response over this range was obtained ($y = 0.010x + 0.22$, $r = 0.998$).

3.3. Analysis of real samples

The tryptophan contents determined in infant formulas, corresponding to the mean values of three replicates, are given in Table 4. In order to ascertain the tryptophan content per gram of protein, the protein content was determined by using the Kjeldahl method [20].

4. Conclusions

The isocratic elution method described here is fast with an analysis time of 25 min with isocratic elution, and the values obtained for the analytical parameters show that the method is sensitive, reproducible and useful for the determination of tryptophan in infant formulas.

Table 2
Reproducibility studies: relative standard deviations for peak areas and retention times of tryptophan

Precision	X_1	S_{n-1}	R.S.D. (%)	X_2	S_{n-1}	R.S.D. (%)
Instrumental[a]	0.68	0.006	0.88	1.93	0.015	0.7
Derivatization[b]	0.72	0.06	8.29	1.93	0.03	1.55
Method[c]	0.82[d]	0.09[d]	11.5	2.00[d]	0.05[d]	2.33

X_1 = Relative area; X_2 = relative retention time; S_{n-1} = standard deviation.
[a] Calculated from eight injections of the same standard (100 nmol) on two different days.
[b] Checked by analysing three standards of the same concentration (100 nmol of tryptophan and 100 nmol of internal standard) twice.
[c] Established by analysis (hydrolysis and derivatization procedure) of three samples (infant formulas) twice.
[d] mg per 100 g.

Table 3
Accuracy of derivatization

Present (mg per 100 g)	Added (mg per 100 g)	Found (mg per 100 g)	Recovery (%)
110 ± 13	466	592	103
		627	111
	188	294	98
			Mean: 104 ± 6

Recovery assays applied to one hydrolysate of infant formula. A known amount of tryptophan was added after the hydrolysis procedure.

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Table 4
Tryptophan contents of infant formulas

Infant formula	Protein content (g per 100 g)	Tryptophan	
		g per 100 g sample	g per 100 g protein
Adapted starting	12.17	0.108 ± 0.011	0.848 ± 0.089
Follow-up	15.86	0.101 ± 0.008	0.639 ± 0.053
Low-birth-mass	15.03	0.099 ± 0.006	0.659 ± 0.042
Without lactose	15.29	0.111 ± 0.021	0.726 ± 0.135
Soy formula	14.42	0.109 ± 0.024	0.754 ± 0.164
Hypoallergenic	14.83	0.079 ± 0.016	0.535 ± 0.109

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