ELSEVIER

Journal of Chromatography B, 657 (1994) 47–52

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Determination of L-asparagine in biological samples in the presence of L-asparaginase

Donatella Gentili[a,*], Massimo Zucchetti[a], Valentino Conter[b], Giuseppe Masera[b], Maurizio D'Incalci[a]

[a]*Mario Negri Institute, Via Eritrea 62, 20157 Milan, Italy*
[b]*Ospedale San Gerardo, Monza (MI), Italy*

(First received December 28th, 1993; revised manuscript received March 11th, 1994)

Abstract

The antileukaemic efficacy of L-asparaginase is related to the ability of the enzyme to induce the complete disappearance from plasma of L-asparagine, an amino acid essential to lymphoblastic leukaemia cells. It is not feasible to monitor L-asparagine plasma levels in patients under L-asparaginase treatment using the usual analytical procedures as the enzyme continues the hydrolysis of L-asparagine after blood samplaing and during plasma extraction. A method was therefore developed for the determination of L-asparagine in patients receiving L-asparaginase. Sulphosalicylic acid is added to blood samples to deproteinize and inactivate L-asparaginase rapidly. The samples are then analysed by HPLC using a Novapack C_{18} column and fluorescence detection. With the same method L-asparagine is determined in blood cells and, by difference, plasma levels are calculated. This method is highly specific and sufficiently simple and sensitive for clinical use.

1. Introduction

L-Asparaginase (L-ASE) is an enzyme that catalyses the hydrolysis of L-asparagine (L-Asn) into L-aspartic acid and ammonia. It is widely used for the treatment of acute lymphoblastic leukaemia (ALL). The antitumour activity of L-ASE has been known since the 1970s and is believed to result from the rapid depletion of L-Asn in plasma [1–4]. Dramatic depletion of plasma L-Asn has in fact been reported [3,5–7] for a long period after treatment with L-ASE.

Asselin *et al.* [8] demonstrated, however, that the measurement of plasma L-Asn in the presence of L-ASE is not accurate unless an enzyme inhibitor is added to prevent the continued hydrolysis of L-Asn. As hydrolysis is very fast even at 4°C, if L-ASE administered to patients is still present in the blood, the determination of L-Asn will not be accurate as the amino acid can still be hydrolysed while the blood is centrifuged to obtain plasma. In this light, previous studies on patients receiving L-ASE probably overestimated the degree and duration of L-Asn depletion. Asselin *et al.* [8] proposed the use of 5-diazo-4-oxo-L-norvaline (DONV) to inhibit L-ASE, but this involves a number of drawbacks that make it not very suitable for routinely monitoring L-Asn levels: (1) DONV is not commercially available and its synthesis is time

* Corresponding author.

0378-4347/94/$07.00 © 1994 Elsevier Science B.V. All rights reserved
SSDI 0378-4347(94)00166-3

consuming (five steps are needed) [9] and requires specific chemical expertise; (2) DONV is very unstable and it is therefore difficult to purify it without its decomposition; (3) the potency of DONV is low and a final concentration of 40 m*M* is required to inhibit L-ASE completely. At this high concentration the problem of impurities becomes substantial, particularly considering that L-Asn itself can be a contaminant. Hence DONV cannot be easily used in routine clinical studies and alternative methods of inhibiting or inactivating L-ASE are needed to monitor L-Asn blood or plasma levels.

In this paper we describe a different method of inactivation for L-ASE by adding sulphosalicylic acid to the whole blood immediately after sampling, in order to obtain a pH < 1, which is suitable for the simultaneous inactivation of L-ASE and the deproteinization of blood. Using this procedure, total blood L-Asn can be determined, then L-Asn can be measured in blood cells and the plasma concentration of L-Asn can be calculated from the difference. The method appears to be sufficiently rapid and reliable for clinical use.

2. Experimental

2.1. Materials

L-Asn and D-homocysteic acid [internal standard (I.S.)], 5-sulphosalicyclic acid, *o*-phthaldialdehyde, iodoacetic acid and propionic acid were purchased from Sigma (St. Louis, MO, USA) and Na_2HPO_4, boric acid and 2-mercaptoethanol from Merck (Darmstadt, Germany). L-ASE, from *Erwinia carotovora*, was obtained from CAMR (Salisbury, UK). Acetonitrile was of HPLC grade from Carlo Erba (Milan, Italy).

2.2. Chromatography

L-Asn levels were measured using an HPLC technique after precolumn derivatization with *o*-phthaldialdehyde as described by Turnell and Cooper [10], using isocratic separation instead of gradient separation. One minute after the derivatization, 25 μl were injected on to a Novapack C_{18} column (150 × 3.9 I.D. mm) with a Resolve C_{18} guard column (Millipore). The flow-rate was 1.6 ml/min.

The amino acids were detected with a fluorescence detector (Applied Biosystems, Ramsey, NJ, USA) using an excitation wavelength of 360 nm and an emission cut-off filter of 418 nm.

The mobile phase was water–acetonitrile–sodium propionate buffer (82:7.5:10.5, v/v). Sodium propionate buffer was prepared as a stock standard solution containing 250 m*M* propionic acid and 350 m*M* anhydrous Na_2HPO_4. The solution was adjusted to pH 6.5 with 2 *M* NaOH. After each injection, the column was washed with water–acetonitrile (70:30, v/v) for 10 min and re-equilibrated for 5 min with the mobile phase before the next injection; the flow-rate was always 1.6 ml/min. These steps of washing and re-equilibration were necessary to remove all other amino acids.

2.3. Standard amino acid solutions

Stock standard solutions of L-Asn and D-homocysteic acid, each 100 μg/ml in 0.1 *M* HCl, were prepared and stored at −20°C. The L-Asn solution was diluted with 0.1 *M* HCl to obtain a calibration graph at concentrations of 1, 2.5, 5, 8 and 10 μg/ml. To 2 ml of each standard solution, 100 μl of 100 μg/ml I.S. solution and 1 ml of saline solution were added, then 50 μl of each standard solution were derivatized as explained above and injected after 1 min. Each concentration for the calibration graph was measured in duplicate.

2.4. Preparation of plasma and blood samples

A 100-μl volume of I.S. solution (100 μg/ml) was added to 2 ml of sample, then 0.5 ml of L-ASE in saline solution was added at different concentrations plus 0.5 ml of 20% (w/v) 5-sulphosalicylic acid. The samples were vortex mixed and, after 1 min, centrifuged for 3 min at 1500 *g*.

The supernatants were frozen at −80°C until analysis. Saline solution was used in place of the solution of L-ASE for tests without the enzyme (control).

2.5. Preparation of cells samples

A 10-ml volume of saline solution was added to 2 ml of blood to wash the cells, then the samples were centrifuged for 10 min at 670 *g* at 4°C, the supernatant being discarded. A 1-ml volume of saline solution was added to reconstitute the sample, followed by 100 μl of I.S. solution (100 μg/ml), 0.5 ml of saline solution and 0.5 ml of 20% (w/v) sulphosalicyclic acid. The samples were vortex mixed and centrifuged for 3 min at 1500 *g* and the supernatants were frozen at −80°C until analysis.

2.6. Quality control

From a volunteer plasma sample we froze aliquots of 2 ml. Each day we used three aliquots to test the reproducibility of the analysis. For each quality control (QC) 100 μl of I.S. solution, 0.5 ml of saline solution and 0.5 ml of 20% (w/v) sulphosalicylic acid were added, then the samples were vortexed mixed and centrifuged for 3 min at 1500 *g*, and 50 μl of the supernatant were derivatized and injected after 1 min.

2.7. Patients

Samples from three patients were analysed to test the feasibility of the method in clinical use. The patients had ALL and were being treated with L-ASE from *Erwinia carotovora*. Two of them were being treated with low doses (10 000 I.U./m^2 every 3 days) and the third with high doses (25 000 I.U./m^2 every 7 days). The patients given low doses were sampled 3 days after the previous dose and patient treated at high doses after 7 days. The samples were handled as described later in Section 3.6.

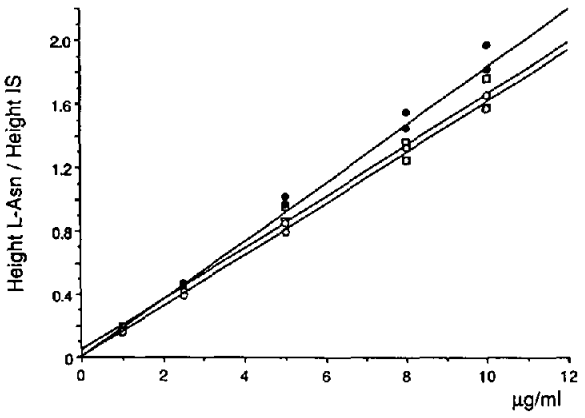

Fig. 1. Calibration graphs for L-Asn constructed on three consecutive days: □ = day 1; ● = day 2; ○ = day 3. Abscissa, concentration of L-Asn in μg/ml; ordinate, height of L-Asn peak/height of I.S. peak.

3. Results

3.1. Calibration

A single-point calibration graph was compared with a multi-point calibration graph (five concentrations) three times on three consecutive days. Fig. 1 shows the multi-point calibration graphs. Each day we also analysed three QCs and calculated their concentrations with both the multi-point and the single-point graphs (10 μg/ml). Table 1 gives the mean and S.D. of the QC concentrations on the three days, calculated with both methods. The difference between the two methods is not significant according to Student's

Table 1
QC concentrations calculated with the multi-point and single-point calibration graphs (10 μg/ml)

Day	Calibration graph	Concentration (mean ± S.D., n = 3) (μg/ml)
1	Multi-point	3.569 ± 0.111
	Single-point	3.568 ± 0.111
2	Multi-point	3.408 ± 0.480
	Single-point	3.462 ± 0.481
3	Multi-point	3.487 ± 0.323
	Single-point	3.644 ± 0.314

t-test. Therefore, the single-point calibration graph was used for further studies.

3.2. Assay to establish whether plasma asparagine could be calculated as the difference between concentrations in blood and blood cells

Six blood samples from healthy volunteers were divided into three aliquots: one to determine L-Asn concentration in blood, one in plasma and one in cells. For a better simulation of the real situation before dividing the samples into aliquots, L-ASE was added to each sample at a final concentration of 30 I.U./ml and left at room temperature for 30 min to hydrolyse plasma L-Asn completely. Then plasma L-Asn was calculated from the difference between the levels in blood and blood cells, considering the haematocrit value, and the difference between the experimental and calculated values of plasma L-Asn were evaluated. The difference was close to zero with an S.D. of 0.5 μg/ml. Therefore, plasma L-Asn can be calculated from the difference between the levels in blood and blood cells, but it is evaluable only if the difference is ≥1 μg/ml (*i.e.* 2 S.D.).

3.3. Absence of interferences and detection limit

To hydrolyse L-Asn specifically, the ability of L-ASE was used. A volume of blood from one volunteer was divided into aliquots of 2 ml, to which 100 μl of I.S. solution were added. Ten samples were sonicated for 5 min, then 0.5 ml of L-ASE was added to seven of them and 0.5 ml of saline solution to three of them as controls (control 1). The concentration of L-ASE was 466 I.U./ml, high enough to ensure complete hydrolysis of L-Asn. Sonication of the samples was necessary to permit L-ASE to hydrolyse the L-Asn inside the cells. All ten samples were then placed in a bath at 37°C for 30 min. Three other samples, prepared as control 1, were deproteinized immediately to check whether ultra-sound treatment caused any changes (control 2). After hydrolysis, the following mean concentrations (± S.D.) of L-Asn in the two groups of controls were found: 11.7 ± 0.9 in control 2 (immediately deproteinized) and 15.4 ± 0.7 in control 1 (after ultra-sound), whereas in the samples with L-ASE the L-Asn peak disappeared (Fig. 2) and was not detectable in any of the seven replicates. Therefore, it can be concluded that the peak is entirely due to L-Asn.

The limit of detection, calculated as the mean concentration of the sample at zero concentration + 2S.D., was 0.2 μg/ml.

3.4. Inhibition of L-ASE

To test the inhibition of L-ASE with sulphosalicylic acid, plasma samples, not blood, were used because L-ASE does not penetrate the cells efficiently so it is impossible to establish the degree of enzyme inactivation.

Three concentrations of L-ASE, 0.05, 0.5 and 1 I.U./ml of plasma, were used. Two controls for each concentration were used, the first without L-ASE and the second with L-ASE, but deproteinized after 5 min before adding sulphosalicylic acid. The second control was used to

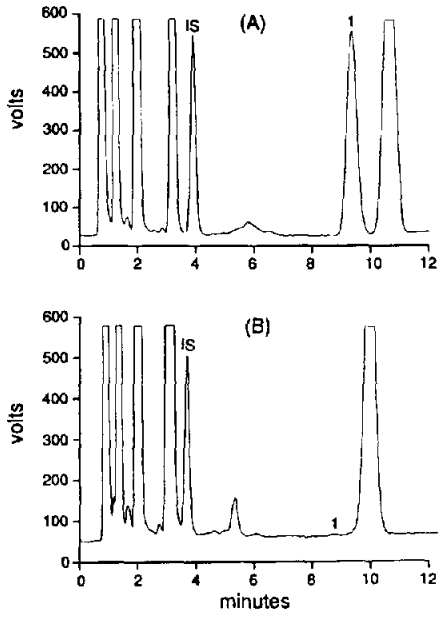

Fig. 2. (A) Chromatogram of a blood sample containing L-Asn (10.7 μg/ml); (B) chromatogram, at the same sensitivity, of the same blood sample after sonication. Peaks: I.S. = internal standard; 1 = L-Asn.

Table 2
L-Asn in plasma samples to which L-ASE was added, deproteinized after 1 min (L-ASE, 1 min) and after 5 min (L-ASE, 5 min)

L-ASI (I.U./ml)	L-Asn (mean ± S.D., $n = 7$) (μg/ml)		
	Controls[a]	L-ASE, 1 min	L-ASE, 5 min
0.05	5.880 ± 0.360	6.060 ± 0.501	3.094 ± 0.318
0.5	5.940 ± 0.480	4.399 ± 0.646	N.D.[b]
1	5.299 ± 0.149	N.D.	N.D.

[a] Controls contained saline solution in place of L-ASE.
[b] Not detectable.

ensure that enough L-ASE was used to hydrolyse plasma L-Asn in 5 min.

Table 2 summarizes the results in terms of mean values and S.D. for the three concentrations of L-ASE. At 0.05 I.U./ml the recovery of L-ASN was excellent (100 ± 8%); at 0.5 I.U./ml the recovery was lower (71.4 ± 13%) and at 1 I.U./ml no L-Asn was recovered.

3.5. Stability of deproteinized samples at −80°C

To 20 ml of blood, 1 ml of I.S. solution and 5 ml of L-ASE (to a final blood concentration of 0.4 I.U./ml) were added. The samples were then deproteinized with 5 ml of 20% (w/v) sulphosalicylic acid, vortex mixed and, after 1 min, centrifuged for 3 min at 1500 *g*. The supernatant was divided into aliquots, frozen and stored at −80°C. On different days, three of them each day were analysed. Table 3 summarizes the results; the samples stored at −80°C were stable for at least 1 month.

Table 3
Concentrations of L-Asn on day 1 and after 1, 2, 3 and 4 weeks

Storage time	Concentration (mean ± S.D., $n = 3$) (μg/ml)
1 day	7.653 ± 0.106
1 week	7.506 ± 0.071
2 weeks	7.273 ± 0.061
3 weeks	7.664 ± 0.108
4 weeks	7.534 ± 0.149

Samples stored at −80°C.

Table 4
L-Asn in blood, cells and plasma of patients with ALL

Patient	Concentration (μg/ml)		
	Blood	Cells	Plasma[a]
1	3.199	2.966	N.D.[b]
2	4.462	2.207	3.221
3	7.624	7.226	N.D.

Patients 1 and 3, treatment at 10 000 I.U./m^2, sampling after 3 days; patient 2, treatment at 25 000 I.U./m^2, sampling after 7 days.
[a] The plasma concentration was calculated from the difference between blood and cell concentrations and refers to 1 ml of plasma considering the haematocrit.
[b] Not detectable.

3.6. Clinical application

From each patient 8 ml of blood were taken and divided into the following parts: 2 ml were deproteinized immediately after sampling, 2 ml were used to obtain cells and 4 ml of blood were centrifuged for 3 min at 1500 *g* to obtain plasma. The samples were then handled as described above and analysed on the same day as sampling. The results are given in Table 4.

In the two patients treated with 10 000 I.U./m^2 L-ASE, L-Asn was undetectable in plasma at the third day because the L-Asn levels in blood are the same as those in cells. Instead, in the patient treated with 25 000 I.U./m^2 L-ASE the calculated plasma level of L-Asn on the seventh day was 3.2 μg/ml.

4. Discussion

L-Asn can be successfully monitored in blood, blood cells and plasma of cancer patients under treatment with L-ASE using the sulphosalicylic acid deproteinization procedure. The method is highly specific and sufficiently sensitive and can be applied in clinical practice as it requires only the addition of sulphosalicylic acid and centrifugation. It is important to remember that blood

must be deproteinized immediately after sampling (within 1–2 min) because waiting even only 5 min can result in significant hydrolysis of L-Asn. Even though the deproteinization of blood cells required a longer time owing to the need for centrifugation and cell separation, this was not a problem because the levels of L-Asn in blood cells are slow to follow the changes in plasma.

This procedure is applicable when the concentration of L-ASE is below 0.5 I.U./ml, so it cannot be used to monitor L-Asn levels just after high doses of L-ASE. However, the purpose of L-Asn monitoring is to assess whether its depletion lasts throughout the interval between doses. According to the available clinical pharmacokinetic data [3], by 3 days after L-ASE doses of up to 25 000 I.U./m^2 the plasma L-ASE is below 0.5 I.U./ml; hence it is advisable to measure L-Asn at least 3 days after the dose of L-ASE to be sure of avoiding the risk of false-negative results.

Acknowledgements

We thank Dr. Luisella Verotta, Department of Organic Chemistry, University of Milan, for the synthesis of DONV. This study was supported by the Tettamanti Foundation.

References

[1] J.D. Broome, *J. Exp. Med.*, 118 (1963) 99.
[2] J.M. Hill, E. Loeb, A. Maclellan, A. Khan, J. Roberts, W.A. Schields and N.O. Hill, *Cancer Res.*, 29 (1969) 1574.
[3] T. Ohnuma, J.F. Holland, A. Freeman and L.F. Sinks, *Cancer Res.*, 30 (1970) 2297.
[4] T. Ohnuma, J.F. Holland and P. Meyer, *Cancer*, 30 (1972) 376.
[5] J.D. Broome, *J. Exp. Med.*, 127 (1968) 1055.
[6] D.A. Cooney, R.L. Capizzi and R.E. Handshumacher, *Cancer Res.*, 30 (1970) 929.
[7] H.K. Miller, J.S. Salser and M.E. Balis, *Cancer Res.*, 29 (1969) 183.
[8] B.L. Asselin, M.Y. Lorenson, J.C. Whitin, D.J. Coppola, A.S. Kende, R.L. Blakley and H.J. Cohen, *Cancer Res.*, 51 (1991) 6568.
[9] R.E. Handshumacher, C.J. Bates, P.K. Chang, A.T. Andrews and G.A. Fischer, *Science*, 161 (1968) 62.
[10] D.C. Turnell and J.D.H. Cooper, *Clin. Chem.*, 28 (1982) 527.