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## Note

# Thin-layer chromatographic determination of L-asparaginase in the presence of human serum

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L-Asparaginase is an aminohydrolase which causes the hydrolysis of asparagine to aspartic acid and ammonia. There is a great deal of interest in studying L-asparaginase from *E. coli* because of the fact that the enzyme has been used extensively for the management of acute lymphocytic leukemia over the last decade [1—5]. The antineoplastic activity is probably due to depletion of circulating pools of asparagine by asparaginase. Despite the therapeutic significance of this enzyme, there is no easy method for its determination.

Assay methods generally measure the enzyme activity either by the determination of ammonia [6] or measurement of NADH formation after coupling aspartate with other enzymes [7—9]. Determination of ammonia by the Nessler reagent [15] is less sensitive and is time consuming. The enzyme coupling methods require the use of coupling enzymes in adequate purity. The radioisotope method based on the separation of aspartic acid from asparagine by ion-exchange chromatography [10] is specific and sensitive but suffers from disadvantages such as being time consuming, lengthy and subject to interferences such as changes in pH.

This paper describes a simple, specific, sensitive and rapid assay for L-asparaginase which is also applicable in the presence of human serum. The enzyme activity is measured by incubating L-asparaginase with the assay mixture and separating the product by thin-layer chromatography (TLC). Under the conditions employed, aspartate moves near the origin, while asparagine moves with the solvent front resulting in a complete separation of both the components.

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## MATERIALS AND METHODS

### *Materials*

Plastic sheets precoated with Ionex SB-Ac were obtained from Brinkman (Westbury, NY, U.S.A.). Prior to use, 1.2 × 9.0 cm strips were cut and equilibrated with 0.05% acetic acid for 30 min as recommended by Devenyi [11]. Unlabelled asparagine, aspartate, and *E. coli* L-asparaginase (Grade 1, 30 U/mg of protein) were obtained from Sigma (St. Louis, MO, U.S.A.). Aqueous counting scintillant was obtained from Amersham (Arlington Heights, IL, U.S.A.). L-[$^{14}$C(U)]Asparagine (150 mCi/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). Due to impurities present in the commercial preparation of radiolabelled asparagine, it was further purified as follows: A 20-$\mu$l aliquot was applied 1 cm from the bottom of the strip (2.5 × 9.0 cm) and ascending chromatography was carried out as described below. Asparagine was eluted from the strip with 1% formic acid. The resin was removed by filtration through a Pasteur pipet plugged with glass wool and the filtrate lyophilised. The residue was dissolved in 0.5 ml of distilled water and lyophilised again. This asparagine was used as a substrate in the assay mixture.

*Procedure for incubation*

Assays were performed at 37° C in a final volume of 0.05 ml in the polypropylene microcentrifuge tubes. The incubation mixture contained 50 m*M* Tris, pH 8.6, 5 m*M* [$^{14}$C]asparagine (800—2000 cpm/nmol). The reaction was initiated by the addition of enzyme to the incubation mixture and terminated by rapidly cooling in ice. In the experiments where human serum was added to the enzyme assays, the final concentration of serum was 20%. The blank usually contained the assay mixture with the denatured enzyme (100° C, 10 min) incubated at 37°C. One international unit of enzyme activity (IU) is equivalent to the amount of enzyme that causes the hydrolysis of 1 $\mu$mol of asparagine per min at 37° C. Specific acitivity is defined as U/mg of protein.

*Thin-layer chromatography*

Radiolabelled aspartate formed by the enzymatic reaction was separated from the unreacted substrate by ascending TLC. Immediately after cooling, 5 $\mu$l of the incubation mixture were spotted and dried at an origin 1 cm from the bottom of the strip. The strip was next placed in a developing tank containing ethyl acetate—acetic acid—water (8:0.05:91.95). After the solvent had migrated about 7 cm from the origin, the strips were removed and dried. Reference strips were also run with unlabelled asparagine and aspartate under identical conditions. Spots corresponding to asparagine and aspartate were cut and placed in the scintillation vials. In cases where reference strips were not run, the strips were cut into 1-cm pieces and placed in separate vials. Radioactivity was determined after eluting amino acids from the resin by adding 3.0 ml of scintillation fluid followed by 0.5 ml of 20% formic acid.

Protein was determined by the dye-binding method using bovine serum albumin as a standard [12].

## RESULTS

### *Separation of aspartate and asparagine*

The separation of asparagine and aspartate with ethyl acetate—acetic acid—water (8:0.05:91.95) in 30 min is seen in Fig. 1. Aspartate moved near the origin and asparagine moved near the solvent front. $R_F$ values of asparagine and aspartate in three separate runs were 0.97 ± 0.02 and 0.04 ± 0.01, respectively. This separation of asparagine from aspartate was used to develop an assay for L-asparaginase.

### *Application to the determination of L-asparaginase*

Fig. 2 shows the separation of aspartate produced by L-asparaginase after incubation with the radiolabelled substrate. Serum alone when assayed under identical conditions did not reveal any enzyme activity. Addition of serum to the assay mixture did not affect the mobility of asparagine or aspartate as a similar separation was obtained when L-asparaginase without any serum was

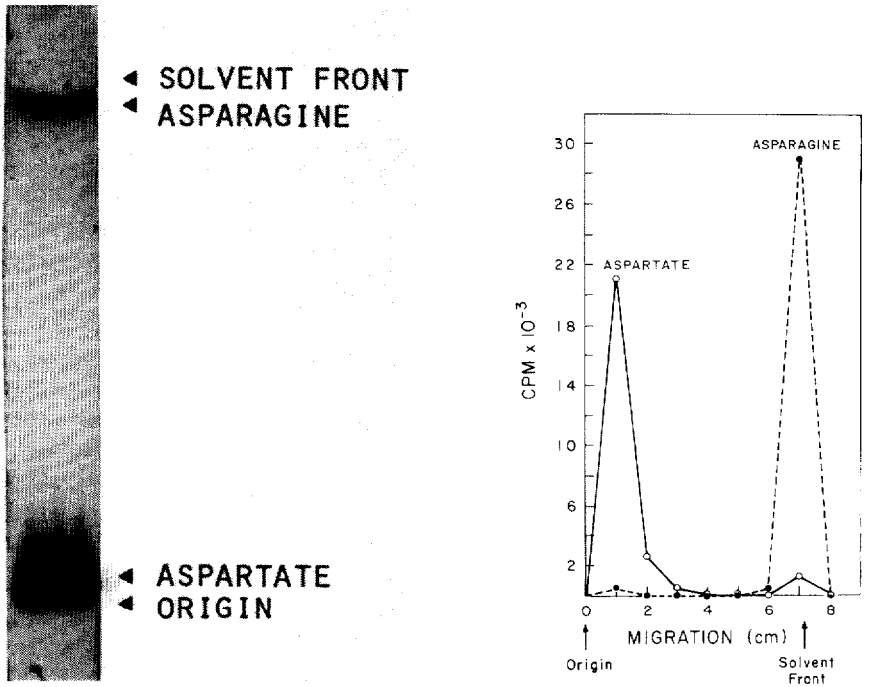


Fig. 1. Separation of asparagine from aspartate. A 2-µl aliquot containing 5 µg of asparagine and aspartate in Tris buffer, pH 8.6, was applied. After development, the strips were dried, sprayed with 0.2% ninhydrin in butanol—acetic acid (95:5) and spots visualised after brief exposure at 70°C.

Fig. 2. Separation of radiolabelled aspartate (○——○) formed from [$^{14}$C]asparagine by 0.8 µg of *E. coli* L-asparaginase added exogenously to a 10 µl of human serum. The assay was performed at 37°C for 10 min and the sample was chromatographed as described in Materials and methods except the strip was cut into 1-cm pieces and radioactivity counted. Mobility of [$^{14}$C]asparagine (●— — —●) on the strip after the assay mixture was incubated with heat-inactivated enzyme at 37°C for 10 min.

used in the assay mixture. The assay also showed a linear relationship for aspartate formation with incubation times ranging up to 30 min, and had a correlation coefficient of 0.998.

*Recovery and reproducibility*

To determine the assay precision, 100 ng and 400 ng of L-asparaginase were added to 10 $\mu$l of human serum. The enzyme activity was determined after incubation with the assay mixture at 37°C for 10 min. The results are shown in Table I. The amount of enzyme added was directly proportional to the amount of enzyme measured.

TABLE I

ACCURACY OF DETERMINATION OF *E. COLI* L-ASPARAGINASE IN THE PRESENCE OF HUMAN SERUM

| Asparaginase added (ng) | Asparaginase found (ng) | Coefficient of variation (%) | Recovery (%) |
|---|---|---|---|
| 100 | 103.1 | | 101.0 |
| | 101.5 | | |
| | 98.4 | | |
| Mean | 101.0 ± 2.43 | 2.40 | |
| 400 | 448.4 | | 102.6 |
| | 382.0 | | |
| | 405.6 | | |
| | 410.4 | | |
| | 418.4 | | |
| | 398.0 | | |
| Mean | 410.5 ± 22.3 | 5.43 | |

DISCUSSION

TLC is a simple, rapid and inexpensive analytical technique for resolving metabolites. Recently, Dunlop et al. [13] used paper chromatography to separate asparagine from aspartate. Aspartate migrated 5—7 cm from the origin, asparagine migrated 10—14 cm from the origin and separation was achieved in 18 h. Pataki [14] lists different solvent systems as to their ability to separate asparagine from aspartate by TLC. However, the differences in $R_F$ values are not large enough to adopt one of these separations as a regular, reliable assay for L-asparaginase. With the present method, aspartate remains near the origin, while asparagine moves with the solvent front and separation is obtained in 30 min.

The present method measures aspartate formation directly and is more sensitive than Nessler's method for ammonia determination or enzyme coupling methods. As little as $4 \cdot 10^{-5}$ IU of L-asparaginase can be detected easily with this method as compared to $1 \cdot 10^{-2}$ IU of enzyme required for Nessler's method or $5 \cdot 10^{-4}$ IU of enzyme required for the enzyme coupling method

[9]. The sensitivity of the present assay was determined using [$^{14}$C]asparagine (2000 cpm/nmol) with 500 cpm as a reliable detectable change in radioactivity. The method could be made more sensitive by increasing the specific activity of the substrate. In comparison to the ion-exchange column method, the present method is simple, rapid, and separation of asparagine from aspartate is not affected by changes in pH of the assay mixture, ionic strength or by constituents present in serum.

In conclusion, we feel that this technique provides a rapid, sensitive and reliable assay for L-asparaginase. The assay should be useful in enzyme clearance studies in patients receiving L-asparaginase therapeutically.

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