Journal of Chromatography, 235 (1982) 249–255
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,267

RADIOISOTOPE ASSAY FOR GLUTAMINE SYNTHETASE USING THIN-LAYER CHROMATOGRAPHY

SHAM L. PAHUJA and TED W. REID*
Yale University School of Medicine, Department of Ophthalmology and Visual Science, New Haven, CT (U.S.A.)

(First received June 1st, 1981; revised manuscript received August 6th, 1981)

SUMMARY

A simple radiochemical method for the determination of glutamine synthetase activity by thin-layer chromatography is described. The assay involves the separation of glutamine from glutamic acid on anion-exchange resin [Dowex 1 (CH_3COO^-)] coated plastic strips. The technique described is fast, reproducible and at least 50 times more sensitive than commonly used colorimetric methods. This method was used to determine the kinetic properties of glutamine synthetase and is applicable with either purified enzymes or crude tissue homogenates. K_M values for glutamic acid, ATP and ammonia determined by the present assay were similar to the values obtained by colorimetric methods.

INTRODUCTION

The enzymatic conversion of glutamic acid to glutamine by glutamine synthetase [L-glutamate: ammonia ligase (ADP), E.C. 6.3.1.2.] occupies a central position in nitrogen metabolism. The presence of this enzyme has been determined in animal tissues, plants, and bacteria. Control and regulation of glutamine synthetase by hormones and various metabolites has been studied in cells grown in tissue culture and purified enzyme preparations[1–7].

Assay methods generally used measure glutamine synthetase activity either by colorimetric[8–10], or radiochemical methods based on the separation of glutamine from glutamic acid by column chromatography[11–13]. Major shortcomings associated with colorimetric techniques are: (1) they do not measure the physiological end products; (2) they lack sensitivity; (3) they are subject to interference by substances which can react with ammonium molybdate or ferric chloride. Radioisotopic methods on the other hand are very sensitive but are time consuming and tedious.

In this paper, we describe a simple, rapid, sensitive and specific assay which avoids the problems associated with the previous assays. The method involves detection and quantitation of glutamine formed from ^3H-labelled glutamic acid by thin-layer chromatography (TLC) on anion-exchange resin [Dowex 1 (CH_3COO^-)] coated plastic sheets. Glutamine and glutamic acid separate well due to the fact that glutamic

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acid is strongly adsorbed while glutamine moves quite readily under the chromatographic conditions.

MATERIALS AND METHODS

Materials

Ionex SB-AC precoated plastic sheets were obtained from Brinkmann (Westbury, NY, U.S.A.). Prior to use, 1.2 × 9.0 cm plastic strips were cut and equilibrated with 0.05% acetic acid for 30 min as recommended by Devenyi[14]. [3,4-^{3}H]glutamic acid (40 Ci/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). ATP, unlabelled glutamic acid and glutamine were obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals and reagents were obtained from the usual sources. Bovine retina was used as a source of glutamine synthetase. Retina was homogenized with 5 m*M* phosphate buffer (pH 7.1), 10 m*M* 2-mercaptoethanol, and 0.1 m*M* EDTA, and was centrifuged at 105,000 *g* for 30 min at 4°C. The supernatant was used as a source of crude enzyme preparation. Glutamine synthetase was purified from the crude extract by ammonium sulfate fractionation followed by conventional chromatographic techniques. The purified enzyme preparation was judged to be homogeneous on the basis of analytical gel electrophoretic techniques under various conditions and immunodiffusion analysis. Details of the enzyme purification and characterization will be published elsewhere[15].

Analytical procedures

The standard incubation mixture (total volume 50 μl, pH 7.4) consisted of: 50 m*M* imidazole, 20 m*M* $MgCl_2$, 10 m*M* ATP, 4 m*M* ammonium chloride, 10 m*M* [^{3}H]glutamic acid (200–1000 cpm/nmole). The reaction was initiated by the addition of an appropriate amount of enzyme preparation. The reaction mixture was incubated at 37°C and stopped by rapid cooling in ice. Immediately after cooling, 5 μl of the incubation mixture were spotted as a streak and dried at the origin 1 cm from the bottom of the strip. Ascending chromatography was carried out in ethyl acetate–pyridine–water (8:1:91) mixture till the solvent approached to within 1 cm of the top of the strip. Areas corresponding to glutamic acid and glutamine, as determined from the reference strips run under identical conditions, were cut and placed in scintillation vials. In cases where reference strips were not run, the strips were cut into 1-cm pieces and individual pieces were placed in separate vials. Amino acids were eluted from the resin by adding 2.5 ml of scintillation fluid (ACS) and 1 ml of 10% formic acid as recommended by Himoe and Rinne[16]. The unit of enzyme activity is expressed as the amount required to convert 1.0 μmol of glutamic acid to product per min at 37°C.

Protein was determined by the method of Bradford[17] using bovine serum albumin as a standard.

RESULTS

Separation of glutamic acid and glutamine

Fig. 1 shows the separation of unlabelled glutamic acid and glutamine with ethyl acetate–pyridine–water mixture (8:1:91) obtained in 30 min. Glutamine moves near the solvent front, while glutamic acid remains near the origin. The contents of

the assay mixture do not significantly affect the separation of glutamic acid and glutamine. As shown in Fig. 2, glutamine formed from radiolabelled glutamic acid by glutamine synthetase was completely separated from the unreacted substrate. Table I shows the R_F values of glutamic acid and glutamine in various solvents. It is evident that as the concentration of acetic acid is increased, mobility of glutamic acid increases withtout any significant effect on mobility of glutamine.

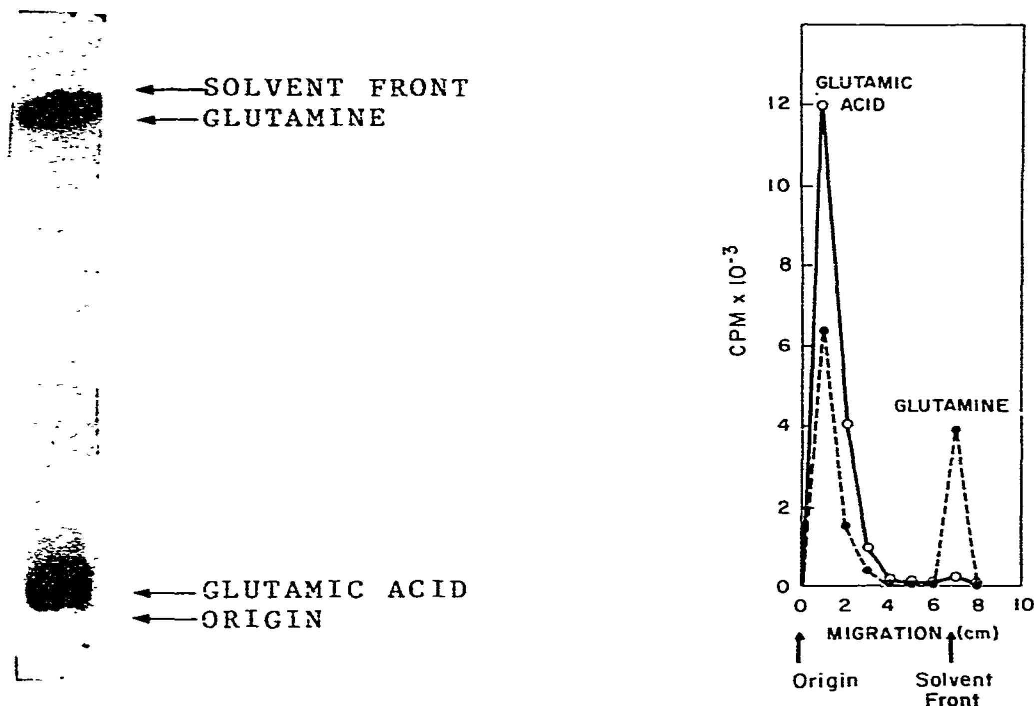

Fig. 1. Separation of glutamic acid and glutamine. A 2-μl aliquot containing 4 μg of glutamic acid and glutamine in imidazole buffer, pH 7.4 was spotted. After development, the strips were dried, sprayed with 0.2% ninhydrin in butanol–acetic acid (95:5) and spots visualized after brief exposure at 70°C.

Fig. 2. Separation of [^{3}H]glutamine (●—●) formed from [^{3}H]glutamic acid on Dowex 1-(acetate) coated plastic sheets. After development, the strips were dried, cut into 1-cm pieces and the radioactivity counted as described in the text. Assay mixture was incubated with 70 μg of crude enzyme protein for 10 min at 37°C. O—O, Migration of [^{3}H]glutamic acid in the presence of assay mixture and enzyme after incubation at 0°C for 10 min. The composition of assay mixture was the same as described in Materials and methods.

Relationship of glutamine synthesis with time and protein concentration

Glutamine synthetase from bovine retina was used to test the linearity of the assay with time and protein concentration. Fig. 3A shows that the amount of glutamine synthesized is linear with respect to time for incubations up to 30 min. The amount of glutamine synthesized was also linear with respect to protein concentration of the crude retinal extract (Fig. 3B). The assay conditions are described under Materials and methods and in the legends to Fig. 3.

TABLE I

R_F VALUES OF GLUTAMIC ACID AND GLUTAMINE IN VARIOUS SOLVENTS

	Solvent	*R_F (Glu)*	*R_F (Gln)*
1	Pyridine-acetic acid-water (0.5:0.2:99.3)	0.21	0.97
2	Pyridine-acetic acid-water (0.5:0.5:99)	0.46	0.97
3	Pyridine-acetic acid-water (0.5:1:98.5)	0.56	0.98
4	Acetic acid-water (0.2:99.8)	0.18	0.97
5	Acetic acid-water (0.5:99.5)	0.38	0.97
6	Acetic acid-water (1:99)	0.49	0.97
7	Methanol-acetic acid-water (10:0.2:89.8)	0.18	0.94
8	Ethyl acetate-acetic acid-ammonium hydroxide-water (5:0.1:0.1:94.8)	0.15	0.86
9	Ethyl acetate-pyridine-water (8:1:91)	0.04	0.98

Requirements for glutamine synthesis

In Table II, the requirements for glutamine synthetase reaction are illustrated. Essentially, no activity was obtained in the absence of ATP, Mg^{2+} or ammonia.

Kinetic characteristics of retinal glutamine synthetase

In order to test the usefulness of this simple procedure in kinetic studies, kinetic characteristics were determined for purified retinal glutamine synthetase. The enzyme shows Michaelis-Menten kinetics when one of the substrates is limiting. Double

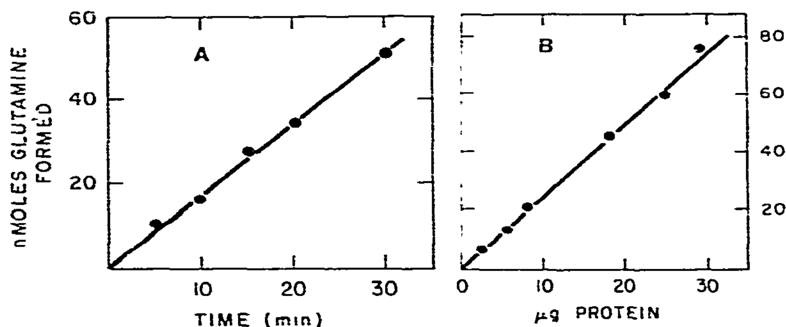

Fig. 3. Relationship of glutamine synthesis with incubation time and protein concentration. A, Effect of various incubation times on product formation using 7.0 μg of crude enzyme. B, Effect of different protein concentrations on product formation during a 10 min incubation time. Each data point represents an average of three determinations. Other details were the same as described in Materials and methods.

TABLE II

REQUIREMENTS FOR GLUTAMINE SYNTHETASE REACTION

The composition of complete system was same as described in Materials and methods.

Reaction conditions	*Relative activity (%)*
Complete	100
Minus ATP	2.9
Minus $MgCl_2$	2.9
Minus NH_4Cl	3.1

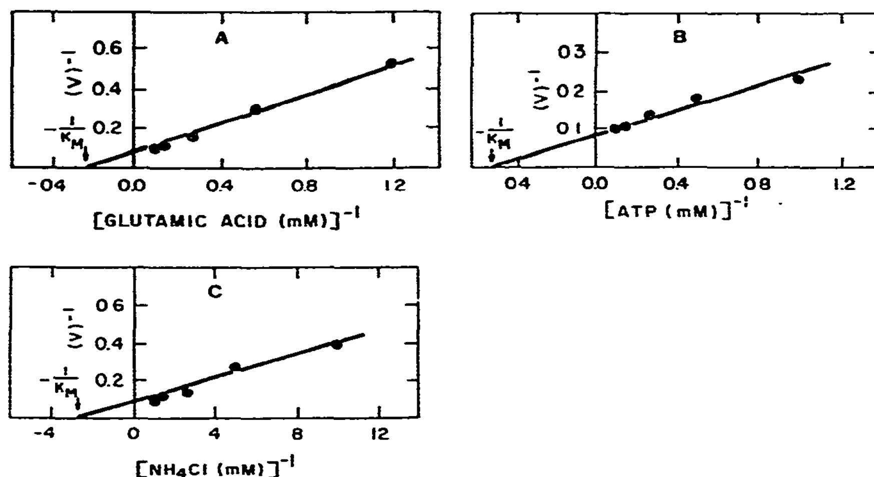

Fig. 4. K_M determination of glutamine synthetase for glutamic acid (A), ATP (B) and ammonia (C). In each case, 0.92 μg of purified glutamine synthetase and a 10 min incubation time were used. Initial velocity is expressed as nmoles of product formed per min. For each determination, the concentration of all substrates except for the one used to determine the K_M was the same as described in Materials and methods for the standard assay.

reciprocal plots of velocity *versus* various concentration of glutamic acid, ATP and ammonium chloride are shown in Fig. 4. Michaelis constants (K_M) for glutamic acid, ATP and ammonium chloride were found to be 3.6 m*M*, 1.9 m*M* and 0.34 m*M*, respectively. K_M values for glutamic acid and ATP were 3.4 m*M* and 2.1 m*M* respectively, by the γ-hydroxyglutamate determination. The K_M value for ammonium chloride was found to be 0.28 m*M* by the inorganic phosphate determination. These values are in quite close agreement with the values obtained by the present assay.

DISCUSSION

The separation of glutamic acid and glutamine is rapidly accomplished by thin-layer chromatography. Different solvent systems were tested as to their ability to separate glutamic acid from glutamine. The solvent system used in the present study had the advantages that both the components move as discrete, compact spots. Further, with this solvent system it is possible to separate γ-aminobutyric acid (GABA) from glutamine. The R_F value of GABA was found to be 0.91 $\pm$ 0.01 from five separate runs in comparison to the R_F value of 0.98 $\pm$ 0.01 for glutamine. This separation can be further increased by increasing development time with the solvent. The identity of glutamine as the only product formed under glutamine synthetase assay conditions has been determined by reversed-phase high-performance liquid chromatography (HPLC) and GABA formed in this reaction constitutes less than 1%[18]. Lack of GABA formation is not due to the absence of active glutamic acid decarboxylase (GAD) in the crude retinal homogenate, as the same retinal preparation is capable of GABA formation under the proper assay conditions. In every case only one product corresponding to either GABA or glutamine was observed depend-

ing upon the assay conditions[18]. The finding of no significant formation of GABA under the glutamine synthetase assay conditions is consistent with previous observations that the ATP and divalent cations used in glutamine synthetase assay are inhibitory for GAD reaction[19–21].

The assay system described here appears to have many advantages over previously used methods. The assay is simple and allows for a rapid determination of multiple samples under controlled conditions. Previous attempts at separation of glutamine from glutamic acid by paper electrophoresis[22] or TLC[23,24] are not as simple to use and small differences in R_F values were seen for the glutamic acid and glutamine under these conditions. Bujard and Mauron[24] reported R_F values for glutamic acid and glutamine of 0.26 and 0.39, respectively. In the present assay, R_F values of 0.04 and 0.98 for glutamic acid and glutamine respectively allow for complete separation of the two compounds.

The measurement of glutamine synthetase by determination of inorganic phosphate[8,9] is subject to interference by substances such as ATP, ADP, AMP, pyrophosphate, proteins and low concentrations of detergents[25–28]. Assays which measure γ-glutamylhydroxamate formation[9,10], are subject to interference by substances that can react with the ferric chloride reagent or glutamylhydroxamate. In addition, another major disadvantage with the hydroxamate method is that enzymes other than glutamine synthetase have been shown to catalyze the glutamate transferase reaction[29] and, hence, any changes in the activity in crude cellular extracts should be interpreted with caution.

Radioisotopic methods involving the use of Dowex 1 (Cl^-)[11], alumina[12], or Dowex 1 (CH_3COO^-) followed by Amberlite CG-50 (H^+)[13] are tedious and time consuming. The elution of glutamic acid and glutamine is sensitive to various factors such as changes in pH, which would cause problems when changes in assay condition are required. Using the strip method we have not observed any changes in the mobility of glutamic acid and glutamine in varying pH of the assay mixture from 6.0 to 8.0.

The present assay is more sensitive than colorimetric methods, measures glutamine formation directly, and is simpler to use than ion exchange columns. With colorimetric methods, it is not possible to accurately determine less than: (1) 50 nmol of γ-glutamylhydroxamate (A_{535} = 0.08)[30] or $5 \cdot 10^{-2}$ units enzyme activity; (2) 100 nmol of inorganic phosphate (A_{625} = 0.07)[31] or $1 \cdot 10^{-1}$ units enzyme activity. Hence, these methods are not sensitive enough to determine low concentrations of enzyme as ordinarily found in cells in culture. Under the present conditions of assay, it is possible to detect the formation of 1 nmole of radiolabelled glutamine ($1 \cdot 10^{-3}$ units enzyme activity) using [^{3}H]glutamic acid (1000 cpm/nmol). Also, it is possible to make the method more sensitive by increasing the specific activity of the substrate. If it is necessary to add other compounds to the assay mixture which might affect the mobility of the product, reference strips can be run under the same conditions, or the strip can also be cut conveniently into 1-cm pieces and the position of glutamic acid and glutamine can be localized accurately. Also, internal standards can be added to the assay mixture and the strip stained with ninhydrin reagent to localize glutamic acid and glutamine before counting.

In summary, we feel that this assay offers several advantages. The procedure is simple, rapid, specific and sensitive and is applicable with a large number of samples. This assay technique is also useful for assaying GAD activity, and is being extended for the determination of glutaminase, aspartate synthetase, and asparaginase.

ACKNOWLEDGEMENTS

We wish to thank Mary Bannon for her help in preparing this manuscript, Brian T. Mullins and Joseph Albert for their technical assistance. This work was supported by National Institutes of Health Grants EY-01656, EY-07000 and EY-00785 and unrestricted funds from Connecticut Lions Eye Research Foundation and Research to Prevent Blindness.

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