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Flow Injection Determination of Glutamate in Human Serum and Rat Brain Samples with Immobilized Glutamate Oxidase and Glutamate Dehydrogenase Reactors[1])

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Summary: Two methods are proposed for the determination of regional concentrations of glutamate in the rat brain as well as in human serum.

Glutamate oxidase was immobilized on non-porous glass beads and glutamate dehydrogenase was immobilized on glass derivatives. These supports were employed for the construction of Single Bead String Reactors and Packed Bed Reactors, respectively, which in turn were linked to Flow Injection Analysis systems with either photometric or fluorometric detection. Analytical working curves are linear in the range 1–200 μmol/l for packed bed reactors and 10–500 mmol/l for single bead string reactors. The samples were pretreated depending on their origin and the applied measuring system. Optimal dilution factors were established for the two techniques. Optimal dilution ratios were established and the influence of several added substances was investigated. Recovery and method comparison studies including high performance liquid chromatography verified the accuracy of the proposed methods. Results from within-day and between-day measurements gave relative standard deviations of 4.7 and 5.9% for serum samples and 2.5 and 4.0% for brain samples, respectively.

Introduction

Glutamic acid is considered to be an important neurotransmitter in the mammalian central nervous system (1) and the suspicion that it may play a significant role in the aetiology and pathophysiology of a number of neurological and psychiatric disorders (2, 3) has generated extra interest in the study of this amino acid. Glutamic acid concentrations are significantly higher in neoplastic ascites than in cardial ascites (4). The increasing trend toward the development of analytical methods for reliable and accurate determination of low glutamic acid concentration in very small samples is therefore not surprising. These methods include enzymatic measurements with glutamate oxidase and glutamate dehydrogenase[2]), either alone (5–7) or in combination with other enzymes (8–11), HPLC (12, 13) and gas chromatography-mass spectrometry (14, 15). All these methods have been applied for the determination of glutamic acid in biological samples with relative success, but they are not exempt from drawbacks such as the use of complicated and/or expensive equipment and the lack of accuracy at low concentrations of glutamic acid in complex matrices. They also are time consuming, tedious and therefore unsuitable for the rapid routine analysis of large numbers of samples.

In this presentation two methods for the determination of glutamic acid in human serum and rat brain were used, based on previous investigations (16, 17).

Materials and Methods

Reagents

All the chemicals used were of analytical-reagent grade and the solutions were prepared in doubly distilled water. Glutamate oxi-

[1]) Funding: STRIDE HELLAS – 33 PROJECT
[2]) Enzymes:
Glutamate dehydrogenase (EC 1.4.1.3)
Glutamate oxidase (EC 1.4.3.11)

dase (EC 1.4.3.1) from *Streptomyces* sp., 5 U/mg solid was a gift from Yamasa Shoyu (Chiba-Ken, Japan). Glutamate dehydrogenase (EC 1.4.1.3) 120 U/mg enzyme protein from beef liver and nicotinamide adenine dinucleotide (NAD^+ grade I, 100%) were purchased from Boehringer Mannheim (Boehringer Mannheim Biochemica, Germany). The rest of the chemical compounds and the enzyme supports (controlled pore glass derivatives and non-porous glass beads) for the immobilization, were purchased from Sigma (St Louis, MO, USA). Glutamic acid standard solutions were prepared from a 0.01 mol/l stock solution by appropriate dilutions.

Instrumentation

Two flow injection analysis systems, using either single bead string reactors consisting of non-porous glass beads with immobilized glutamate oxidase, or packed bed reactors with immobilized glutamate dehydrogenase on Aminopropyl-controlled pore glass and isothiocyanate-controlled pore glass, were used in connection with photometric or fluorometric detection, depending on the employed enzyme. In the second of these enzymatic sytems the NADH generated is proportional to the original concentration of glutamic acid and is measured fluorometrically at 460 nm after its excitation at 340 nm. Three values were obtained for each sample, one spectrophotometrically and two fluorometrically, since the two controlled pore glass reactors were used separately in the flow injection analysis. The flow injection measurements were performed with a home made unit used in two different arrangements. The flow injection analysis set-up consists of a four-way pneumatically actuated injection valve, an eight channel peristaltic pump and a home made filter spectrophotometer or a spectrofluorimeter (RF-551, Shimadzu, Japan) equipped with low volume flow-through cells (2 µl and 12 µl, respectively). The experimental set-ups, which are shown in figure 1, can be thermostated with a controlled water bath. Data collection and processing was performed with an IBM-compatible personal computer and a software written in Microsoft Q-Basic. The interface unit was an RTL 800/815 multifunction input-output board.

Preparation and treatment of the samples

The rats were killed by decapitation and the brains were removed. The middle brain, the cerebral cortex, the hypothalamus and the striatum together with *substantia nigra* were dissected and rapidly dropped into liquid nitrogen to minimize post mortem changes. Amounts of 25–30 mg of the sample were homogenized in 2.0 ml of 80% cold ethanol in water (normally used for proteinaceous tissues) under ultrasonication at 45 W for three minutes. The suspension was centrifuged for 10 min at 1370 *g*, then the supernatant combined with the washings (3.5 ml total volume) was concentrated to dryness under reduced pressure. To the residue were added 1 ml of 0.1 mol/l phosphate buffer pH 8.0 and the resultant suspension was filtered through a 0.45 µm membrane filter to remove the particulate matter.

The human serum samples were obtained from patients of the Ioannina University Hospital. Three different methods for the deproteinization of serum samples were employed including either chemical or physical techniques.

Method (a): Serum sample (500 µl) was mixed with 80 µl of a 150 g/l zinc sulphate and 300 µl of a 50 g/l barium hydroxide. The mixture was centrifuged and the supernatant was analysed after appropriate dilution and ultrafiltration through a membrane with a nominal weight cut off of M_r 30 000 (Millipore, Bedford, MA 01730 USA) for fluid clarification. An excess of the reagents was necessary for the completion of the precipitation of the proteins. The time needed to prepare the filtrate was 10 min.

Method (b): Serum sample (400 µl) was applied to a Sephadex G-25 superfine column (Pharmacia, Uppsala, Sweden, 1.5 cm × 4.8 cm). The glutamic acid was eluted with buffer solution, 0.1 mol/l phosphate pH 8.0. The analyte appears quantitatively between 2.5 and 8.0 ml of the elution volume. This procedure takes only about 5 min.

Method (c): The serum samples were diluted 1 : 20 or 1 : 7 (depending on the applied method, photometric or fluorimetric) with phosphate buffer and proteins were removed by ultrafiltration through a membrane with a nominal weight cut off of M_r 10 000. This procedure takes about 15 min for the preparation of 300 µl of a protein-free solution.

Analytical procedure

In the case of photometric detection the procedure entails injection of the sample into the carrier stream and the production of H_2O_2 on the single bead string reactor containing the immobilized glutamate oxidase. The reaction mixture meets the *Trinder* reagent (18) at the output of enzymatic reactor and flows through a second single bead string reactor (40 cm × 0.8 mm), filled with untreated glass beads, which allows sufficient time for the completion of the reaction. The maximum of the absorbance-time curve, A_{max}, is measured automatically at 510 nm (fig. 1). The working pH of the system is 7.8, the over-all flow rate 0.25 ml/min and the concentrations of aminoantipyrine, 3,5-dichloro-2-hydroxybenzenesulphonic acid and peroxidase are 0.4 mmol/l, 1.2 mmol/l and 440 000 U/l, respectively. The absorbances measured for the construction of the calibration curve were in the range 0.030–0.850. The optimal working conditions as well as the specificity of the analytical method have been well established in a previous publication (16).

When fluorescence detection is used, the circulating solution with the sample is driven to a 15 cm mixing single bead string reactor positioned after the intake of NAD^+. The mixture then passes through the immobilized glutamate dehydrogenase reactor. The peak height of the fluorescence curve is used for the quantification of glutamic acid. The optimal pH of the system is 8.0, the overall flow rate 0.32 ml/min and the final concentration of NAD^+ 3.2 mmol/l. A detailed description of the immobilization procedure is given elsewhere (17).

The sampling throughput for both systems is 40 samples per hour and the required volume for each replicate is 55 µl.

Results and Discussion

The enzymatic methods depend on the specificity of the enzyme and the secondary reactions involved. Table 1 shows the results of the interference study with 15 different compounds. Glutathione, uric acid, ascorbic acid (and other reducing compounds, data not shown) interfere seriously with the photometric method as they usually do in all procedures involving peroxidase-hydrogen peroxide systems (19). Ascorbic acid is partly auto-oxidized in phosphate buffer to dehydroascorbic acid. The response of the fluorimetric system is not affected by the presence of α-ketoglutaric acid, NH_4^+ and folic acid in concentrations reported in table 1, since their normal concentrations in human serum are ca. 6 µmol/l, 80 µmol/l and 30 nmol/l, respectively (20).

Deproteinization of the samples was necessary in order to avoid any high blanks and double peaks during the measurements. Most of the protein precipitants either cause undesirable effects that decrease the lifetime of the reactors, or they cause proteolysis with release of

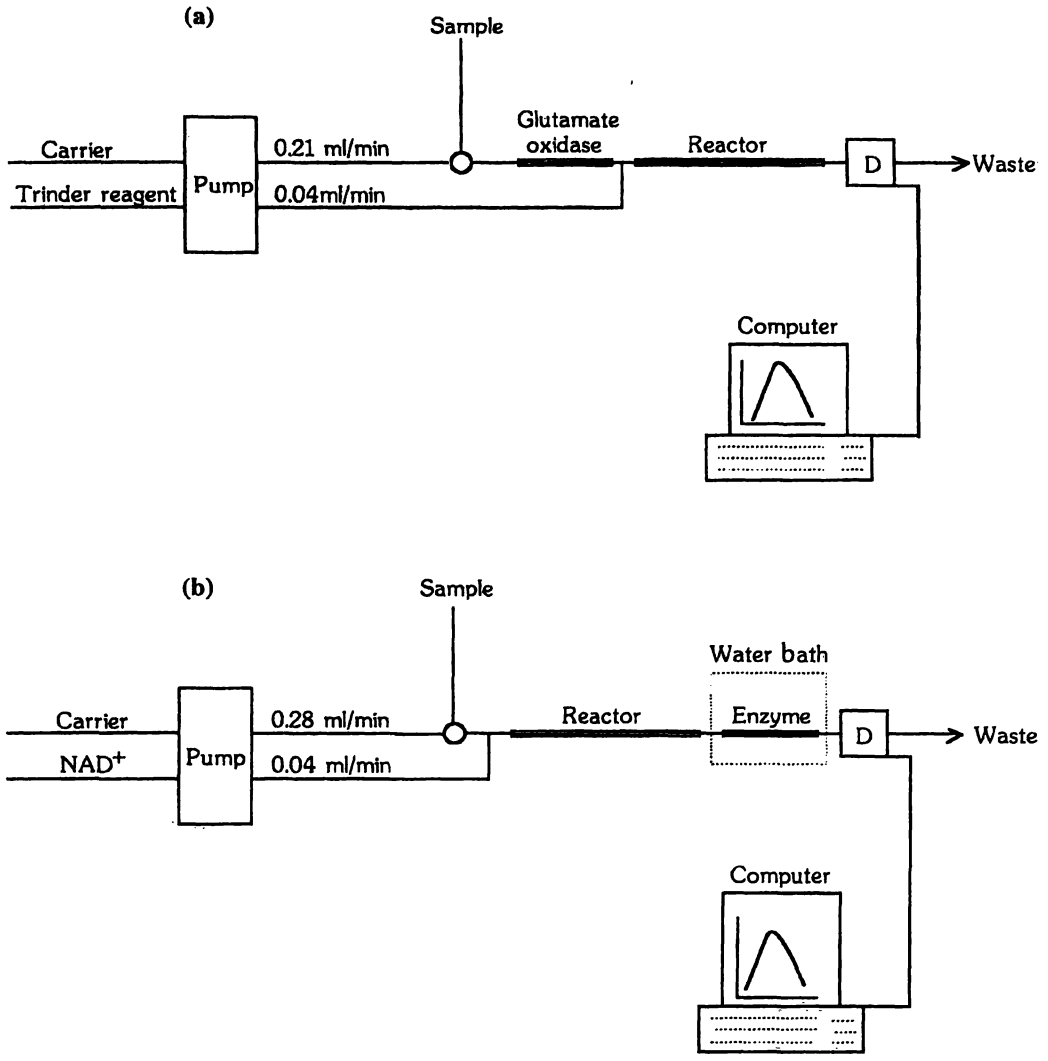

Fig. 1 Flow injection assay set-up for glutamic acid determination:
(a) Manifold for photometric detection,
(b) Manifold for fluorometric detection,
Reactor: Single Bead String-Plain Reactor (i.e. reactor filled with untreated glass beads),
D: Detector, Enzyme: glutamate dehydrogenase.

amino acids, which therefore rise to abnormal concentrations in the serum samples. Precipitation with sulphosalicylic acid causes losses of about 20% for all amino acids (21) while the background absorbance in the filtrates reaches its highest value during precipitation with trichloroacetic acid and sodium tungstate (22).

The well established coprecipitation (23) applied in method (a) was further exploited during our work: efficient removal of proteins and the non-glucose reducing compounds is a critical condition for the implementation of the photometric method and the use of single bead string reactor bearing the glutamate oxidase. Glutamate dehydrogenase on the other hand, is susceptible to Ba^{2+} and Zn^{2+} interference.

Ultrafiltration and gel filtration are simpler and faster approaches for the preparation of protein-free solutions. The dilutions of the serum samples prior to their ultrafiltration reduces significantly the time for one analysis, thus minimizing the probability of hydrolysis of serum proteins or of glutamine breakdown (24). The final preparation was ultrafiltered and determined by the fluorometric method. In this case, ultrafiltration proved to be an ideal technique and the recovery of the added glutamic acid was almost complete.

Based on the results of the above mentioned investigation, the precipitation with zinc sulphate was applied for the determination of glutamic acid in serum samples taken from seven adult patients. All alternative methods for the removal of proteins from the serum samples proved to be unsuccessful. An explanation for this is probably the presence of reducing interferences in most of the samples. In contrast, the above assay was feasible, because ultrafiltration and gel filtration remove any optical interference prior to the fluorometry. The results given in table 2 substantiate the work described above. The gel filtration procedure, which results in a dilution

Tab. 1 Effects of several compounds on the assay of glutamic acid. Standard solutions of glutamate, 100 μmol/l for the photometric and 50 μmol/l for the fluorometric manifold, were used. The concentration of each tested compound was 1 mmol/l.

Interfering compound	Relative response	
	Photometric method	Fluorometric method
None	100	100
L-Methionine	100	103
L-Norvaline	100	112
γ-Aminobutyric acid	100	100
Heparin	102	102
Citrate	101	82
Cholesterol	100	98
Urea	98	99
Uric acid	71	99
Glutathione	46	104
Glutamic ester[a]	100	102
Peptide 1[b]	101	105
Peptide 2[c]	101	106
Folic acid[d]	100	103
α-Ketoglutaric acid[e]	100	98
Ammonium ion[f]	100	99

[a] HGlu(OMe)OH
[b] Glu-Ile-Asn-Arg-Pro-Asp-Tyr-Leu-Asp-Phe-Ala
[c] Ala-Asn-Pro-Glu-Ala-Glu-Gly-Gly-Leu-Glu-Trp-Leu-Asn-Arg-Arg-Ala
[d] 100 nmol/l
[e] 30 μmol/l
[f] 300 μmol/l

Tab. 2 Determination of glutamic acid in serum samples by the two proposed methods and the HPLC method, in mmol/l. The results for the fluorometric methods were obtained after ultrafiltration of the samples. All the results are the average of triplicates and the errors range from 0.01 to 0.03 SD.

Sample	Method			
	Photometric Single bead string reactor	Fluorometric		HPLC (15)
		Aminopropyl-controlled pore glass	Isothiocyanate-controlled pore glass	
1	0.18	0.19	0.18	0.19
2	0.12	0.12	0.12	0.11
3	0.39	0.38	0.39	0.39
4	0.17	0.17	0.17	0.16
5	0.37	0.37	0.37	0.37
6	0.36	0.37	0.38	0.37
7	0.21	0.22	0.21	0.23

of the sample 1 : 13.75, gave results very close to the obtained with the above procedure. If necessary, higher dilutions were performed, to attain almost undetectable blank values in the flow injection measurements.

In the case of brain tissue no protein removal or any other treatment was necessary, since no blank values were observed after the dilution of the liquid sample extracted by the method given above. The photometric method does not suffer from the above mentioned interferences. Table 3 gives results of the regional concentration of glutamic acid obtained by applying the two methods. All the concentrations of glutamic acid found in the serum samples and these from different regions of brain are in good agreement with those obtained by the HPLC method, omitting the hydrolysis step (25).

The within-day results and between-day reproducibility studies in two representative samples (serum sample and brain tissue) gave the relative standard deviations for the two methods which appear in table 4.

The accuracy of the proposed methods was also verified by recovery studies, in which standard glutamic acid solutions were added to several samples. Recoveries of 93−104% were attained as shown in table 5.

The proposed methods can also be applied for the determination of glutamic acid in blood plasma after adding heparin as anticoagulant to the blood samples.

In conclusion, the two proposed analytical procedures seem to be sensitive, reproducible, accurate and relatively rapid for the determination of glutamic acid in serum and brain tissue. The assay can be carried out with simple and easily available instrumentation. Compared with the well known HPLC methods, our methods offer simplicity and low cost, and they are well suited to the routine analysis of large numbers of samples (40 against 2 samples per hour), without any derivatization. The reactors are stable when kept in a refrigerator in buffered phosphate, when not in use. They can be used for more than 3000 runs and retain 75% of their initial activity after a working period of three months. The detection limits of the proposed methods are comparable

Tab. 3 Glutamic acid concentrations in μmol/g of wet tissue measured in several regions of the rat brain. The data presented are the means of triplicate measurements by each of the proposed methods. The SD of the means ranges from 0.1 to 0.3.

Region	Method			
	Photometric Single bead string reactor	Fluorometric		HLPC (15)
		Aminopropyl-controlled pore glass	Isothiocyanate-controlled pore glass	
Cerebral cortex	10.1	9.3	9.1	–
Hypothalamus	6.3	6.5	6.7	6.6
Middle brain	12.0	12.5	12.7	12.4
Striatum-Substantia nigra	9.8	10.3	10.3	–

Tab. 4 Within-day and between-day relative standard deviations (CV) for the two methods and two representative samples.

Sample	Within-day CV (%)		Between-day CV (%)	
	Photometric method	Fluorometric method	Photometric method	Fluorometric method
Serum (0.37 mmol/l)	5.1 (n = 7)	4.0 (n = 7)	6.0 (n = 6)	5.8 (n = 6)
Middle Brain (12 μmol/g)	2.4 (n = 5)	2.7 (n = 5)	4.4 (n = 4)	3.6 (n = 4)

Tab. 5 Recovery of *L*-glutamic acid added to the serum and brain samples analysed in tables 2 and 3, respectively.

Sample, unit	Method						
	Photometric				Fluorometric with Aminopropyl-controlled pore glass		
	Added mmol/l	Taken* mmol/l	Found mmol/l	Recovery %	Taken* mmol/l	Found mmol/l	Recovery %
Serum 1, mmol/l	0.15	0.33	0.31	94	0.34	0.34	100
Serum 7, mmol/l	0.20	0.41	0.38	93	0.42	0.41	98
Hypothalamus, μmol/g tissue	5.0	11.3	11.3	100	11.5	11.4	99
Middle Brain, μmol/g tissue	7.0	19.0	19.7	104	19.5	19.9	102

* These concentrations represent the values given in tables 2 and 3 for the respective samples, plus the added amounts (given under "added" in the table).

with those of the majority of the HPLC techniques (almost 0.3 μmol/l) and adequate for the applications given above. The enzymatic methods with the soluble enzymes are not automated and they are uneconomic, because of the irreversible consumption of the enzymes.

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