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Short communication

High-performance liquid chromatographic analysis of β-phenylethylamine for the estimation of in vivo protein synthesis

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Abstract

A rapid, sensitive, and automated reversed-phase liquid chromatographic method was developed for the analysis of phenylalanine as β-phenylethylamine, for the measurement of in vivo protein synthesis. β-Phenylethylamine was derivatized with *o*-phthaldialdehyde (OPA) to form a fluorescent derivative that was successfully measured in tissue cell fluids and hydrolysates as the decarboxylation product of phenylalanine. The system was extremely sensitive enabling the accurate determination of 0.5 pmol in biological samples. Analysis time was less than 11 min, so that 130 samples can be analysed per day. The method eliminates the need for time-consuming column extraction procedures. This method offers substantial advantages over existing methods for the isolation and determination of β-phenylethylamine.

1. Introduction

The "flooding-dose" technique is considered to be the method of choice for measurement of the rate of protein synthesis in vivo [1]. The method, developed by Garlick et al. [2], involves pulse injection of a large dose of radiolabelled (^{3}H or ^{14}C) and unlabelled phenylalanine. This floods and rapidly equilibrates all phenylalanine pools in the body and maintains the phenylalanine specific radioactivity relatively constant over the period of radiolabel incorporation. The simplicity of the flooding-dose technique has led to its wide use for measuring protein synthesis in vivo [1,3,4].

Determination of the amount of phenylalanine in order to determine its specific radioactivity in large numbers of tissues has remained problematic. [^{3}H]Phenylalanine, in vivo, is converted to [^{3}H]tyrosine, which necessitates the isolation of phenylalanine. This can be achieved by enzymatic decarboxylation of phenylalanine to β-phenylethylamine followed by organic extraction

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[2]. The amount of β-phenylethylamine present in the organic extract is determined by spectrofluorometric analysis of a ninhydrin derivative of β-phenylethylamine [5]. Unfortunately, the ninhydrin derivative is highly unstable in the presence of light or heat, making this method time-consuming, labour intensive and not suitable for automation.

In this paper, we describe a rapid, sensitive, and automated high-performance liquid chromatographic (HPLC) method that we routinely use for the measurement of phenylalanine as β-phenylethylamine for the determination of in vivo protein synthesis.

2. Experimental

2.1. Materials

HPLC-grade water and methanol, Brij 35 and tetrahydrofuran were obtained from Fisher Scientific (Edmonton, Alb., Canada). *o*-Phthaldialdehyde (OPA), 2-mercaptoethanol, L-tyrosine decarboxylase (T4379), ethanolamine, and β-phenylethylamine hydrochloride were purchased from Sigma (St. Louis, MO, USA). L-[2,6-^{3}H]Phenylalanine was obtained from Amersham Canada (Oakville, Ont., Canada). Radiochemical purity was checked using column or paper chromatography. Radiochemical purity was greater than 99%.

2.2. Animals and tissue sampling

Male Sprague–Dawley rats (Charles River, St. Constant, Que., Canada) were housed in individual cages and had free access to food and water in compliance with the guidelines of the Canadian Council on Animal Care. Each rat was injected with 150 μmol phenylalanine per 100 g body weight, containing 50 μCi L-[2,6-^{3}H]phenylalanine. After 15 min, rats were killed by cervical dislocation and the soleus, extensor digitorum longus and gastrocnemius muscles and the liver were immediately removed and placed in liquid nitrogen and stored at −50°C until analysed.

2.3. Preparation of tissue samples

Tissue samples were prepared in a manner similar to that reported by Garlick et al. [2]. Briefly, muscles (40 to 500 mg) and 500 mg liver were homogenized in 2–3 ml of 2% $HClO_4$ using a glass-to-glass tissue homogenizer. They were then centrifuged at 2800 *g* for 15 min at 0°C. Half a volume of saturated potassium citrate was added to the supernatant, which was then centrifuged at 2800 *g* for 15 min. Supernatants were used for the estimation of free phenylalanine specific radioactivity. The protein precipitate was washed four times with 8 ml of 2% $HClO_4$ and then hydrolysed in 5 ml of 6 *M* HCl for 24 h at 110°C. HCl was removed by vacuum centrifugation and the amino acids were resuspended in 1 ml of 0.5 *M* sodium citrate at pH 6.3. The hydrolysate was used to determine protein-bound phenylalanine specific radioactivity. Phenylalanine was converted to β-phenylethylamine by adding 0.25 ml of L-tyrosine decarboxylase (2 units/ml) suspended in 0.5 *M* sodium citrate (pH 6.3), containing 1 mg of pyridoxal phosphate per ml, to 1 ml of supernatant or 0.5 ml of hydrolysate. The samples were incubated for 17–20 h in a waterbath at 50°C.

The extraction of β-phenylethylamine was performed in a manner similar to that reported by Suzuki and Yagi [5] and Garlick et al. [2]. β-Phenylethylamine was isolated by adding 0.5 ml of 3 *M* NaOH and 5 ml of chloroform–*n*-heptane (1:3, v/v) to the incubated sample. The sample was shaken for 2–3 min, then centrifuged for 5 min at 200 *g* and the organic (upper) layer removed. The organic layer was shaken with 2.5 ml of chloroform and 2.0 ml of 0.05 *M* H_2SO_4. The aqueous (top) layer was then placed in a 65°C shaking water-bath for 30 min to remove any residual chloroform because residual chloroform could result in pipetting problems. The aqueous layer was saved for HPLC and liquid scintillation counting. The efficiency of extraction of β-phenylethylamine was 60% with a coefficient of variation of 1.5% ($n = 6$).

Chloroform was either freshly boiled or distilled. We found the efficiency of extraction of β-phenylethylamine varied greatly depending

upon the batch of chloroform. We surmized this to be due to trace quantities of phosgene, a breakdown product of chloroform that forms following its exposure to heat or light. Phosgene reacts with amine groups resulting in non-polar products, which would cause losses of β-phenylethylamine. Because phosgene boils at 8°C and chloroform at 61°C, we boiled or distilled the chloroform, which eliminated the problems with the solvent extraction.

2.4. HPLC analysis

Ethanolamine was used as an internal standard in all samples. Stock solutions of β-phenylethylamine (25 nmol/ml) and ethanolamine (25 nmol/ml) were used for calibration.

Samples containing 100 μl of supernatant or 50 μl of hydrolysate (diluted in water 1:100), 50 μl of internal standard, 100 μl of saturated sodium borate, and 1.0 ml of water were prepared and placed in 2.0-ml vials in an autosampler at room temperature.

The HPLC system consisted of a Varian Model 5000 liquid chromatograph coupled to a Varian Fluorochrom fluorescence detector (excitation 340 nm, emission 450 nm). Derivatization of β-phenylethylamine was performed using OPA reagent, originally used for amino acid analysis by Jones and Gilligan [6]. The fluoraldehyde reagent was prepared by dissolving 0.5 g *o*-phthaldialdehyde in 12.5 ml of HPLC-grade methanol, 0.5 ml of 2-mercaptoethanol, 2 ml of Brij 35, and 112 ml of 0.04 *M* sodium borate (pH 9.5). A Shimadzu autosampler (Model SIL-9A) (Shimadzu Scientific Instruments, Columbia, MD, USA) was used to add and mix 250 μl of OPA reagent solution with the sample–internal standard mixture and to inject 25 μl of the derivatized solution onto the column. A C_{18} reversed-phase Supelco column (Supelcosil LC-18 reversed-phase; 75 × 4.6 mm I.D.; particle size, 3 μm) (Bellefonte, PA, USA) protected by a guard column (50 × 4.6 mm I.D.; particle size, 40 μm) was used for all samples. Two solvents (solvent A: tetrahydrofuran–methanol–0.1 *M* sodium acetate, 5:95:900, v/v, pH 7.2; solvent B: methanol) were used to form the following gradient: 40% B at 0 min to 90% B at 4 min, 90% B at 4 min to 40% B at 4.5 min, and 40% B from 4.5 to 10.3 min. The flow-rate was 1.5 ml per min. Peak areas and retention times were measured using a Shimadzu EZchrom chromatography data system.

3. Results

Using the conditions described, the retention times of ethanolamine and β-phenylethylamine were 2.8 and 6.4 min, respectively. Fig. 1A illustrates the relative fluorescence response of the β-phenylethylamine derivative and the

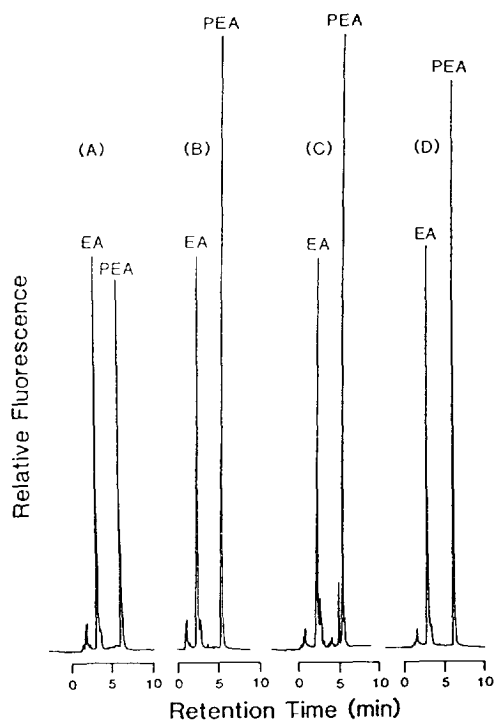

Fig. 1. Chromatograms obtained from standards and biological samples incubated with tyrosine decarboxylase. (A) Standard containing 33 pmol β-phenylethylamine (PEA). (B) Muscle tissue fluid from the extensor digitorum longus. (C) Hepatic tissue fluid. (D) Muscle protein hydrolysate from the extensor digitorum longus. All samples were chromatographed on a 3 μm Supelcosil LC-18 column (75 × 4.6 mm I.D.) at a flow-rate of 1.5 ml per min; 26 pmol of the internal standard ethanolamine (EA) was added to all samples.

ethanolamine derivative when 33 and 26 pmol were injected on the HPLC column, respectively. Serial dilution of β-phenylethylamine standards resulted in a linear relative fluorescence response from 8 to 840 pmol per injection. The coefficient of variation for 25 pmol of β-phenylethylamine was 2.1% ($n = 6$) using six different HPLC runs on the same day. As little as 0.5 pmol β-phenylethylamine can be accurately determined in biological samples (Fig. 2); our criterion for the detection limit was when peak areas were five times the background noise.

Fig. 1B–D illustrate that β-phenylethylamine was successfully isolated from muscle and liver cell fluid and muscle hydrolysates after their incubation with tyrosine decarboxylase and solvent extraction. The chromatograms in Fig. 1 illustrate that not all compounds containing a primary amine were removed during the organic extraction. Standards containing tyrosine, tyramine, and phenylalanine were analysed and their retention times did not correspond to any of these compounds. Since these compounds are not present in these fractions, they do not introduce additional radioactivity that could interfere with the calculation of phenylalanine specific radioactivity. Furthermore, using the present system, β-phenylethylamine was stable for at least 64 h at room temperature permitting samples to be stored in an autosampler. Therefore, with a total analysis time of 10.3 min, over 130 samples can be analysed per day.

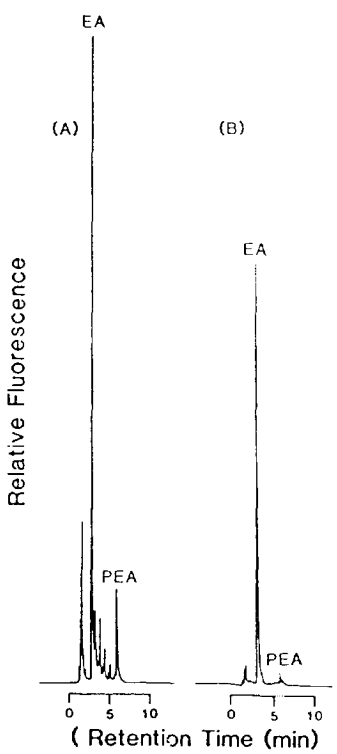

Fig. 2. Chromatograms of supernatant fluid not incubated with tyrosine decarboxylase. (A) Liver containing 40 pmol of endogenous β-phenylethylamine (PEA). (B) Muscle containing 0.5 pmol endogenous β-phenylethylamine (PEA); 26 pmol of the internal standard ethanolamine (EA) was added to all samples and standards.

4. Discussion

Our objective was to develop a rapid, sensitive, and automated method for the determination of β-phenylethylamine, specifically for the measurement of in vivo protein synthesis. Our method represents a substantial improvement over the original method of Suzuki and Yagi [5]. The fluorometric ninhydrin derivative is unstable in the presence of light and heat and the analysis time must be kept constant using a stopwatch, making the analysis time-consuming, inconvenient and unsuitable for automation. Samples must also be incubated for one hour to form the derivative. As a result, β-phenylethylamine can be determined on only 30–40 samples each day. In addition, the method is relatively insensitive with a detection limit of 100 pmol β-phenylethylamine and the efficacy of the solvent extraction cannot be monitored.

In our method, problems that may arise from an unstable OPA derivative are minimized because the autosampler is used to form the derivative, which maintains a constant time between derivatization and injection. There are no lengthy derivatization procedures because the autosampler performs this function, which occurs in less than 2 min. As a result, β-phenylethylamine can be determined in over 130 samples each day. In addition, chromatograms can be monitored for the presence of contaminants that

would contribute to the overestimation of phenylalanine specific radioactivity. In practice, we have found this useful due to the occasional improper solvent extraction, when the organic layer becomes contaminated with the aqueous layer from the first extraction. This method is very sensitive; as little as 0.5 pmol β-phenylethylamine can be accurately determined. Although not specifically utilized in this study, the sensitivity of the present system may make it suitable for the measurement of endogenous β-phenylethylamine, a neurotransmitter present in trace quantities, in biological samples (Fig. 2).

A limiting feature of the original ninhydrin-spectrofluorometric method [5] for measuring phenylalanine during the estimation of protein synthesis is that a relatively large proportion of the sample is required to determine the amount of β-phenylethylamine, leaving a relatively small proportion for determining radioactivity or for repeat measurements [1]. The increased sensitivity of our method permitted the use of only 100 and 50 μl of supernatant and hydrolysate, respectively. The modifications to the solvent extraction can increase the radioactivity and total β-phenylethylamine four to six fold. Our method potentially results in the need to use less radiotracer for measuring protein synthesis.

A variety of other methods are available for the analysis of β-phenylethylamine [7–11]. However, none of these methods were intended for the analysis of β-phenylethylamine for the purpose of measuring protein synthesis. These methods include the use of negative-ion gas chromatography–mass spectrophotometry [7], electron-capture gas chromatography [8,9], and other HPLC methods [10,11]. All these methods are more labour intensive and were designed for the measurement of endogenous β-phenylethylamine as a trace neurotransmitter. As a consequence, laboratories measuring in vivo protein synthesis have not adopted any of these methodologies as evidenced by a lack of citations in the literature. Many laboratories [3,4,12,13] measuring in vivo protein synthesis using the flooding-dose technique of Garlick et al. [2] still use the original ninhydrin method of Suzuki and Yagi [5].

Some investigators [14,15] have avoided the problems with the ninhydrin method by measuring phenylalanine directly by HPLC. Phenylalanine in hydrolysate and homogenate supernatants is separated from other amino acids using ion-exchange chromatography and is then detected with a florescence detector. Effluent fractions are collected and the radioactivity associated with the phenylalanine peak is measured by liquid scintillation counting. However, analysis times are much longer than in our method. In order to have sufficient radioactivity for liquid scintillation counting, a large amount of sample must be injected onto the column, which can result in decreased column life as well as problems with amino acid detection. These systems are also more complicated, which would require more technical expertise to run and maintain.

Therefore, our method using the high resolving capability and sensitivity of the HPLC technique, coupled with full automation and short analysis time, offers substantial advantages over previously developed methods for the isolation and determination of β-phenylethylamine for the measurement of in vivo protein synthesis in large numbers of samples.

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