Journal of Chromatography, 567 (1991) 49–56
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5839

Determination of γ-glutamyl conjugates of monoamines by means of high-performance liquid chromatography with electrochemical detection and application to gastropod tissues

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(First received September 26th, 1990; revised manuscript received January 3rd, 1991)

ABSTRACT

Catabolism of aminergic neurotransmitters in gastropods appears to be primarily by means of γ-glutamyl conjugation rather than by oxidative deamination as is typical of vertebrates. High-performance liquid chromatography with electrochemical detection was used to develop a method for the routine measurement of γ-glutamyl conjugates of dopamine and 5-hydroxytryptamine in gastropod tissues.

INTRODUCTION

Considerable attention has been given to the use of gastropod nervous tissue as a model for the study of aminergic neurotransmission [1–3]. While several aspects of aminergic neurotransmission are similar in molluscs and vertebrates, recent evidence suggests that the catabolism of amines by gastropods is considerably different from that determined in vertebrates [4–6]. Examination of gastropod tissue preparations has suggested a general absence of acidic monoamine metabolites [4,7,8], monoamine oxidase activity [9] and the presence of other metabolites tentatively identified as glucuronide [4] or γ-glutamyl [5,6] conjugates of dopamine (DA) and 5-hydroxytryptamine (5-HT). In particular, evidence now suggests that rather than using monoamine oxidase to inactivate monoamines, gastropods [5,6] and *Limulus* [10] use conjugation with glutamic acid.

Alternate routes of monoamine catabolism are not unprecedented. Vertebrates are known to use methylation through catechol methyl transferase [11,12] and sulphation [13] to inactivate a portion of their monoamines while insects have been demonstrated to use N-acetylation and sugar conjugation to catabolise amines [14,15]. The fact that gastropods may use γ-glutamyl conjugation of amines as the major catabolic route for these compounds necessitates the re-evaluation of experiments which used pharmacological perturbations specific for classical monoamine systems. For example, the validity of experiments where

0378-4347/91/$03.50

monoamine transmission in gastropods is assumed to be enhanced or altered by the administration of monoamine oxidase inhibitors must now be questioned, given the apparent lack of monoamine oxidase activity. Recognition of these alternate routes for amine catabolism will ultimately lead to a better understanding of invertebrate models of aminergic neurotransmission.

High-performance liquid chromatography with electrochemical detection (HPLC–ED) is the current method of choice for measuring DA, 5-HT and their related metabolites in vertebrates [16–18], insects [14,19,20], mollusks [21] and other tissues [22,23]. We now use synthetic γ-glutamyl conjugates of DA and 5-HT to demonstrate that the major metabolites of DA and 5-HT by preparations of gastropod tissue are indeed the γ-glutamyl conjugates of these amines. Further we present a simple, accurate, sensitive and quantitative method for determining the concentrations of these catabolites as well as the native amines in gastropod tissues using α-glutamyl-DA or isoproterenol as an internal standard. The method also confirms that the monoamine oxidase metabolites dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA) are not major catabolic products of DA and 5-HT in gastropods.

EXPERIMENTAL

Animals

An inbred lab-reared strain of the pond snail, *Helisoma trivolvis*, was used. Snails were maintained under flow-through conditions on a 12 h–12 h light-dark cycle. They were fed lettuce or trout chow daily [24].

Tissue preparation

DA and 5-HT were administered as injections into the body cavity. Doses of drugs were 5 μg/g body weight and were administered in 25-μl volumes per gram body weight. After 30 min snails were decapitated, removed from their shells, and all tissues placed in preweighed 1.5-ml polypropylene microtubes and immediately frozen on dry ice. Tissues were stored frozen at − 28°C until estimation of amine concentrations (usually less than one week). Amines and related metabolites were extracted by homogenising tissues in 2 ml of 0.2 *M* perchloric acid containing 0.1 m*M* sodium metabisulphite, 0.25 m*M* EDTA and 1.0 μg/ml α-glutamyl-DA as internal standard. Tissues were homogenized using an ultrasonic tissue disrupter. Homogenates were centrifuged for 10 min in a desk top centrifuge (Fisher Model 235A) and 50 μl of the supernatant injected directly onto the HPLC column.

Conditioned media was made by incubating whole *Helisoma* brains in 50% Liebowitz-15 media (Helisoma Liebowitz-15; Gibco, special order [25]) at two brains per milliliter. The media were incubated for a total of six days, and every 24 h a 150-μl aliquot was removed and assayed for DA, 5-HT and their metabolites; 150 μl of conditioned media were combined with 30 μl of 0.2 *M* perchloric

acid containing 0.1 m*M* sodium metabisulphite, 0.25 m*M* EDTA and 1.0 μg/ml α-glutamyl-DA as internal standard. A 50-μl aliquot was then injected directly onto the HPLC system.

Chromatography

Concentrations of amines and related metabolites were determined by HPLC–ED. The standard separation of amines was achieved using conditions similar to those previously described [6]. The mobile phase consisted of 75 m*M* NaH_2PO_4, 1 m*M* sodium octylsulphate, 0.05 m*M* EDTA and 13% (v/v) acetonitrile. The pH was adjusted to 2.65 with concentrated phosphoric acid, degassed and pumped at a flow-rate of 0.8 ml/min. The HPLC–ED apparatus consisted of a Gilson Model 305 pump equipped with a Model 805 manometric unit. In order to minimise flow perturbations a Scientific Systems Model LP-21 LO-PULSE pulse dampener was placed prior to the injector (Gilson, Model 231 sample injector equipped with a 50-μl sample loop). Separations were achieved using a 250 mm × 4.6 mm I.D. analytical column packed with octadecyl-bonded spherical 5-μm silica particles (Ultrasphere, Beckman) and protected by a 30 mm × 4.6 mm I.D. guard column (MPLCRP-18 SPHERI-5, Brownlee). All separations were achieved at room temperature. DA, 5-HT, DOPAC, 5-HIAA, γ-glutamyl-DA, γ-glutamyl-5HT, α-glutamyl-DA and isoproterenol (ISP) were detected by electrochemical oxidation using a Bioanalytical Systems Model LC-4B thin-layer amperometric detector which was controlled at 0.675 V *versus* an Ag/AgCl reference electrode. Detection of γ-glutamyloctopamine and γ-glutamyltyramine was accomplished by elevating the detector potential to 0.95 V. Signals for the detector were integrated by peak height using a Hewlett-Packard Model HP3394A integrator.

Hydrodynamic voltammograms were produced under standard conditions by altering the applied potential in 0.05-V increments, while detector linearity was determined under standard conditions using a range of standard concentrations from 0.1 to 50 ng in 50 μl. The effect of mobile phase pH on the retention coefficients of a number of amines and their metabolites was achieved by reducing the pH of the standard separation conditions to 2.6 with concentrated phosphoric acid and gradually elevating the pH to 3.50 with concentrated sodium hydroxide. The column was equilibrated for 30 min after each pH change.

Pharmacological agents and chemicals

DA · HCl, 5-HT creatinine sulphate, 5-HIAA, DOPAC and ISP were purchased from Sigma (St. Louis, MO, U.S.A.). γ-Glutamyl-DA · 1/2 C_2H_5OH, γ-glutamyl-5-HT · 1/2 C_2H_5OH · H_2O, γ-glutamyl-*p*-octopamine acetate, γ-glutamyl-*p*-tyramine and α-glutamyl-DA hemihydrate were generously provided by Dr. J. S. Kennedy and the Chemical Synthesis Program of the National Institute of Mental Health.

All chemicals used for analyses were reagent grade or better and all solvents were HPLC grade. Water was distilled and deionised with a resistance of greater than 17 MΩ.

RESULTS AND DISCUSSION

Fig. 1 shows typical chromatograms of DA, 5-HT, their acidic and γ-glutamyl conjugates in standards (A), in snail tissues after the injection of DA and 5-HT (5 μg/g) (B) and in media in which snail ganglia have been incubated for 96 h (C). The γ-glutamyl conjugates were well separated from interfering compounds as well as the acidic metabolites DOPAC and 5-HIAA. No α-glutamyl-DA was present in normal tissue samples, and as a result, this compound can be used as an internal standard although ISP can also be used. Injection of large amounts of DA into snails sometimes produced, in addition to the large γ-glutamyl-DA peak, a small peak with retention characteristics similar to α-glutamyl-DA. This indicates that caution sould be exercised when using α-glutamyl-DA as an internal standard in certain situations. Recovery of amines and their conjugates was excellent. Recovery of either ISP or α-glutamyl-DA added to samples prior to homogenisation was essentially 100% from media and in excess of 90% from tissue homogenates.

Dopamine, DOPAC and γ-glutamyl-DA possess similar electrochemical characteristics (Fig. 2). This also holds true for 5-HT, 5-HIAA and γ-glutamyl-5-HT (Fig. 2). This indicates that these γ-glutamyl or acidic substitutions have little effect on the electrochemical characteristics of DA or 5-HT under these separative conditions. It also indicates that if γ-glutamyl-DA cochromatographed

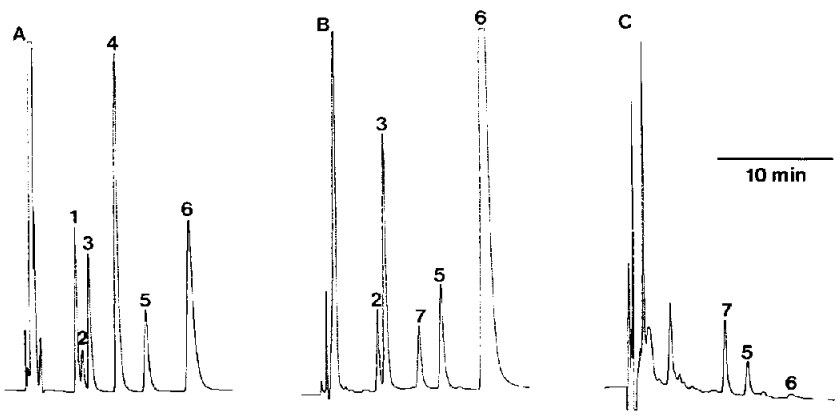

Fig. 1. Example chromatograms of 12.15 ng standards of dopamine, 5-hydroxytryptamine and related metabolites (A), extract of snail tissue 30 min after injection of a mixture of dopamine and 5-hydroxytryptamine (B) and extract of conditioned medium 96 h after addition of snail nervous tissue (C). Chromatogram B demonstrates the production of γ-glutamyldopamine and γ-glutamyl-5-hydroxytryptamine from the injected chemicals and chromatogram C demonstrates the release of γ-glutamyl-5-hydroxytryptamine and 5-hydroxytryptamine into the medium. Peaks: 1 = dihydroxyphenylacetic acid; 2 = γ-glutamyldopamine; 3 = dopamine; 4 = 5-hydroxyindoleacetic acid; 5 = γ-glutamyl-5-hydroxytryptamine; 6 = 5-hydroxytryptamine; 7 = α-glutamyldopamine. The α-glutamyldopamine is used as an internal standard. Amplification of the detector signal is 50 times greater in chromatogram C than in A or B.

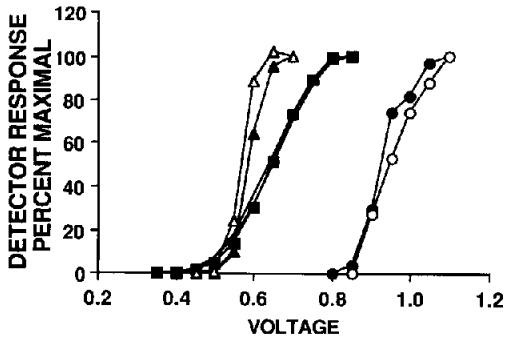

Fig. 2. Hydrodynamic voltammograms of dopamine, 5-hydroxytryptamine and some related acidic and γ-glutamyl metabolites; 10 ng of synthetic standards were measured under standard separation conditions. (■) Dopamine (coincident with dopamine is dihydroxyphenylacetic acid and γ-glutamyldopamine); (▲) 5-hydroxytryptamine (coincident with 5-hydroxytryptamine is 5-hydroxyindoleacetic acid); (△) γ-glutamyl-5-hydroxytryptamine; (●) γ-glutamyltyramine; (○) γ-glutamyloctopamine.

with DOPAC or γ-glutamyl-5-HT cochromatographed with 5-HIAA it would be very difficult to distinguish these compounds on the basis of electrochemical characteristics.

Detector response (peak height) was linear over a wide range of concentrations (data not shown) with detection limits for the γ-glutamyl conjugates of DA and 5-HT close to 50 pg in 50 μl.

The retention of γ-glutamyl-DA, α-glutamyl-DA and γ-glutamyl-5-HT was much more pH-dependent than that of DA, 5-HT, DOPAC, 5-HIAA or ISP (Fig. 3). The optimal pH for the separation of all of these compounds is pH 2.65 although, as DOPAC and 5-HIAA do not appear to occur in snail tissues, other pH ranges can be used without the possibility of interference from these compounds.

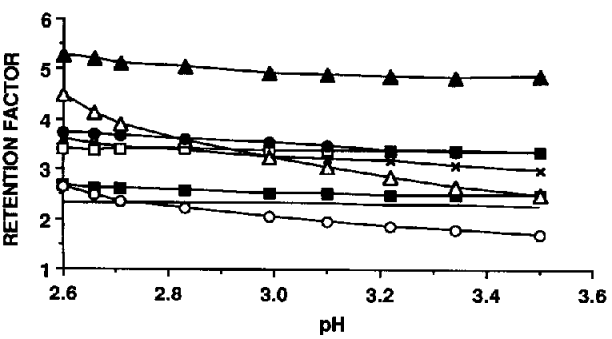

Fig. 3. Effects of pH on the retention characteristics of dopamine, 5-hydroxytryptamine and some metabolites. Values are the retention time divided by the time of initiation of the solvent front. (■) Dopamine; (▲) 5-hydroxytryptamine; (—) dihydroxyphenylacetic acid; (□) 5-hydroxyindoleacetic acid; (○) γ-glutamyldopamine; (×) α-glutamyldopamine; (△) γ-glutamyl-5-hydroxytryptamine; (●) isoproterenol.

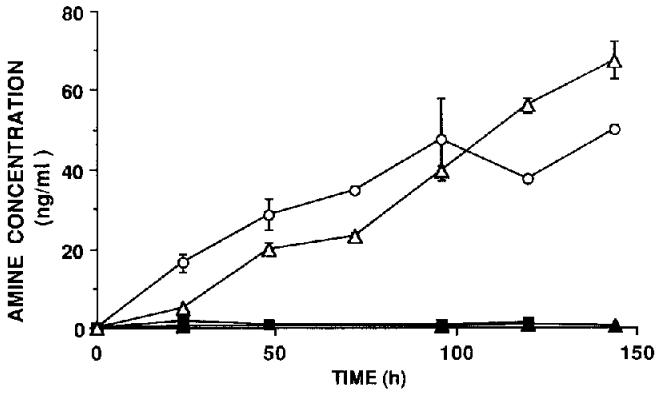

Fig. 4. Time course for the release of dopamine, 5-hydroxytryptamine and their γ-glutamyl conjugates into media in which snail ganglia are incubated. Values are the means ± the standard error of the means derived from two samples assayed in duplicate. (■) Dopamine; (▲) 5-hydroxytryptamine; (○) γ-glutamyldopamine; (△) γ-glutamyl-5-hydroxytryptamine.

The γ-glutamyl metabolites of DA and 5-HT accumulate in incubation media in which snail ganglia are maintained (Fig. 4). Small amounts of DA and 5-HT are also observed in these media but the major metabolites observed are γ-glutamyl-DA and γ-glutamyl-5-HT. No detectable DOPAC, α-glutamyl-Da or 5-HIAA accumulates in incubation media in which snail ganglia are maintained.

Injection of either DA or 5-HT into snails for 30 min results in the elevation of both the parent amines and their γ-glutamyl conjugates (Table I). No detectable DOPAC or 5-HIAA was observed and a small amount of what may have been α-glutamyl-DA was observed in DA injected animals.

TABLE I

EFFECTS OF INJECTION OF DOPAMINE (DA) OR 5-HYDROXYTRYPTAMINE (5-HT) (5 μg/g) ON DA, 5-HT, γ-GLUTAMYL-DA AND γ-GLUTAMYL-5-HT CONCENTRATIONS IN SNAIL TISSUE

Values are the means ± the standard error of the means based on five determinations.

Treatment	Concentrations (ng/g)			
	DA	γ-Glutamyl-DA	5-HT	γ-Glutamyl-5-HT
Saline injection, 30 min	195 ± 40	110 ± 35	351 ± 92	173 ± 28
DA, 5 μg/g, 30 min	2124 + 369	1130 ± 417	234 ± 18	116 ± 15
5-HT, 5μg/g, 30 min	174 ± 9	123 ± 28	1909 ± 174	618 ± 105

Both incubation of nervous tissue from and injection of DA and 5-HT into *Helisoma trivolvis* indicate that this gastropod, like *Aplysia* [5] and *Helix aspersa* [6], uses conjugation with glutamic acid as a major route for the catabolism of endogenous and exogenous amines. This is also in agreement with other researchers who have examined *Limulus* tissues [10]. The conversion of amines to γ-glutamylamines occurs through the action of a γ-glutamylamine synthetase [10] and is thought to be a pathway for amine inactivation [5]. As γ-glutamyl conjugation interferes with the availability of the terminal amine group, which is usually required for recognition of aminergic neurotransmitters, the proposal that γ-glutamyl conjugation is an inactivation mechanism is reasonable. In addition, the fact that the γ-glutamyl conjugates of amines are less lipophylic than the amines themselves suggests that they can be excreted easily.

Octopamine and tyramine are also considered important monoamine candidates for neurotransmitter in mollusks and other invertebrates [26,27]. The present HPLC–ED method can measure both these amines and their γ-glutamyl conjugates. The retention factor (retention time of the compound divided by the time to the initiation of the solvent front) under standard conditions is 2.43 for γ-glutamyloctopamine and 4.07 for γ-glutamyltyramine and detection limits for both compounds are in the order of 200 pg. High electrochemical potentials are required when using amperometric detectors to measure γ-glutamyloctopamine and γ-glutamyltyramine (Fig. 2). These high potentials often result in rapid loss of detector sensitivity through oxidation of the cell surface. Use of coulometric detectors with screening capabilities [28] should provide a simple and reliable HPLC–ED method for the measurement of γ-glutamyl conjugates of octopamine and tyramine derived from gastropod tissues.

HPLC–ED provides a simple and reliable method for the determination of γ-glutamyl conjugates of DA, 5-HT and other neurotransmitter amines. The method should be applicable to investigations of amine catabolism and γ-glutamylamine synthetase activity in grastropods and may be useful for studies involving other invertebrates.

ACKNOWLEDGEMENTS

The authors would like to thank the Alberta Heritage Fund for Medical Research (B.D.S.) and the Natural Sciences and Engineering Research Council of Canada Grant No. U0553 (J.I.G.) for funding of this research. We would also like to thank Dr. R.E. Peter for his continuing support through Natural Sciences and Engineering Research Council of Canada Grant No. A6371 and his interest in this work. We thank Dr. J. S. Kennedy and the Chemical Synthesis Program of the National Institute of Mental Health for the gift of γ-glutamyldopamine · 1/2 C_2H_5OH, γ-glutamyl-5-hydroxytryptamine · 1/2 C_2H_5OH · H_2O, γ-glutamyl-*p*-octopamine acetate, γ-glutamyl-*p*-tyramine and α-glutamyldopamine hemihydrate.

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