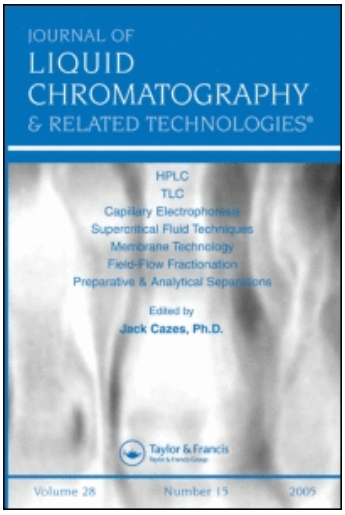

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Determination of Biogenic Amines and Related Compounds in the Ganglia and the Auricle and Ventricle of the Heart of the Snail Helix Lucorum L. by HPLC with Amperometric Detection

A. Pappa-Louisi[a]; E. Rofalikou[b]; B. Michaelidis[b]
[a] Laboratory of Physical Chemistry Department of Chemistry, Aristotle University of Thessaloniki, Thessaloniki, Greece [b] Laboratory of Animal Physiology Department of Biology, Aristotle University of Thessaloniki, Thessaloniki, Greece

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DETERMINATION OF BIOGENIC AMINES AND RELATED COMPOUNDS IN THE GANGLIA AND THE AURICLE AND VENTRICLE OF THE HEART OF THE SNAIL HELIX LUCORUM L. BY HPLC WITH AMPEROMETRIC DETECTION

A. Pappa-Louisi,[1,*] E. Rofalikou,[2] B. Michaelidis[2]

[1] Laboratory of Physical Chemistry
Department of Chemistry
Aristotle University of Thessaloniki
54006 Thessaloniki, Greece

[2] Laboratory of Animal Physiology
Department of Biology
Aristotle University of Thessaloniki
54006 Thessaloniki, Greece

ABSTRACT

Biogenic amines and related compounds in the suboesophageal ganglia and the auricle and ventricle of the heart of the snail Helix lucorum L. have been identified and quantified using the technique of reversed phase ion-pair HPLC with amperometric detection. HPLC analysis revealed the presence of two catecholamines, Dopamine (DA) and Norepinephrine (NE), one indoleamine, Serotonin (5HT), its immediate precursor 5-Hydroxytryptophan (5HTP) and their metabolites 3,4-Dihydroxy phenylacetic acid (DOPAC) and 5-Hydroxyindole-3-acetic acid

(5HIAA). Quantitatively, DA and 5HT were the most prominent monoamines detected, with the ganglia containing the highest concentrations of both. The ganglia contained also assayable amounts of NE and DOPAC.

INTRODUCTION

Biogenic amines, such as the indoleamine serotonin and the catecholamine dopamine, were found to act as central neurotransmitters in molluscs[1] and, also to be implicated in the control and regulation of the cardiac activity in gastropods and bivalves.[2] However, progress in research in these fields requires accurate data detailing the levels of monoamines and monoamine metabolites and precursors in nervous and non-nervous tissues of these species. In the last decade, high performance liquid chromatography (HPLC) with amperometric detection became one of the most important techniques for the transmitter research,[3-6] since it is a sensitive, precise, rapid, and relatively simple technique for the determination of very small amounts of these endogenous compounds in biological tissues.

The aim of the present study is: 1) to investigate the retention behaviour of catecholamines (DA, E, and NE), indoleamines (5HT), their precursors (DOPA, TRYP, and 5HTP) and their main metabolites (DOPAC, 3MT, HVA, 5HIAA, and 5HTOH), see Figures 1 and 2, in reversed phase liquid chromatography (RPLC), 2) to find optimum conditions for their separation, and 3) to detect the above compounds in the suboesophageal ganglia and in the auricle and the ventricle of the heart of the snail Helix lucorum L.using HPLC with electrochemical detection.

EXPERIMENTAL

Chromatography

The liquid chromatography system consisted of a Shimadzu LC-9A solvent delivery unit with a double-plunger reciprocating pump, a Model 7125 syringe loading sample injector fitted with a 20 μL loop (Rheodyne, Cotati, CA), and a Gilson Electrochemical detector (Model 141). The working electrode was a small disc of glassy carbon (3mm diameter) and the electrode

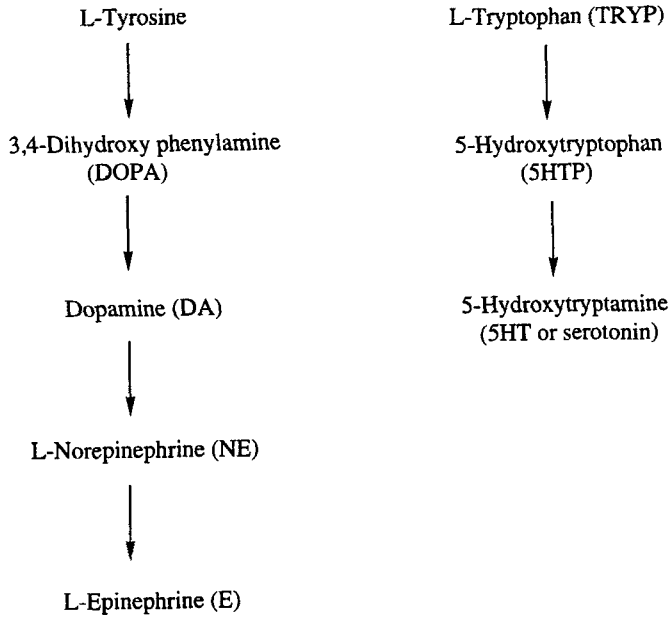

Figure 1. Biosynthetic pathway of the catecholamines and an indoleamine from their precursor aminoacids.

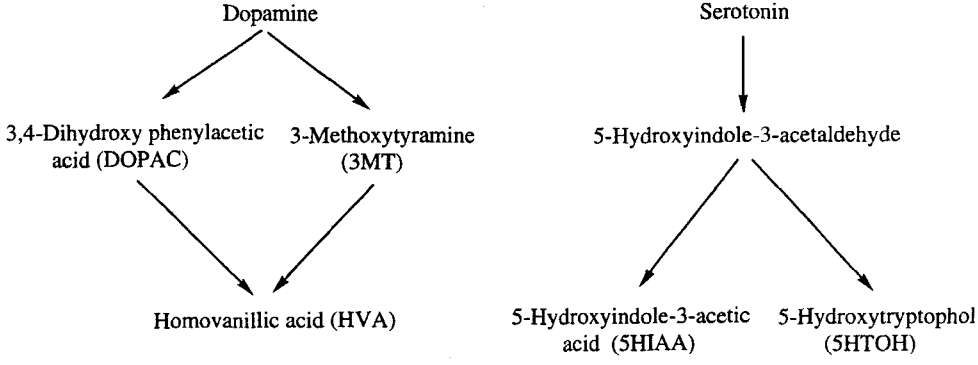

Figure 2. Metabolic pathway of Dopamine and Serotonin.

potential was maintained at 0.65 V vs a Ag/AgCl (3.0 M NaCl) reference electrode. The electrochemical detector was interfaced to a PC 486 IBM compatible computer via a 14-bit AD-DA card. The same computer was also used to carry out all calculations reported in this paper.

The mobile phase was a mixture of an aqueous buffer (plus an ionic modifier) with an organic solvent. The aqueous buffer (pH=3.3) consisted of 0.085 M sodium acetate, 0.09 M tartaric acid, and 1.0 mM Na_2EDTA. Methanol was used as an organic modifier and sodium octyl sulphonate (SOS) as an ion-pairing agent. The methanol and SOS concentration, as well as the chromatografic column, are specified in the Results and Discussion section. The mobile phase was filtered through a mixed esters membrane filter (0.45μm Schleicher & Schuell GmbH, Germany), sonicated and degassed under vacuum. The mobile phase was run at a flow rate 1.0 mL/min and was recycled. All separations were carried out at ambient temperatures.

Chemicals

All chemicals were used, as received, from commercial sources. NE hydrochloride, E and DA hydrochloride were obtained from Aldrich Chemical Co. (Milwaukee, WI). DOPA, 3MT hydrochloride, 5HT creatine sulfate complex, 5HTP, DOPAC, 5HIAA, HVA, TRYP, 5HTOH, Nω-Methylserotonin (M5HT) oxalate, 3,4-Dihydroxybenzylamine (DHBA) hydrobromide, and Epinine (EPI) hydrochloride were available from Sigma Chemicals Co. (St. Louis, MO). All other chemicals were of analytical-reagent grade.

Standards and Sample Preparation

Stock solutions of the analytes and internal standards were prepared in the aqueous buffer of the mobile phase at a concentration of 0.1mg/mL and stored at 4°C. Standard solutions (0.32-0.032μg/mL) were prepared at weekly intervals by an appropriate dilution of the stock solutions using the same buffer as the diluent.

The suboesophageal ganglia as well as the auricles and the ventricles of the hearts were excised out by removing the shells. These tissues after dissection were pooled separately to give samples of 0.01-0.04 g and stored at -70°C until immediately prior to analysis. Then, the samples were thawed and homogenized in the aqueous buffer containing an internal standard, using

a Potter-Elvehjem homogenizer. Then the homogenates were centrifuged at 20,000 r.p.m. for 10 min and, in the end, aliquots of the clear supernatants were either injected immediately onto the chromatographic column or kept at 4°C for up to 48 h.

RESULTS AND DISCUSSION

Retention Behaviour and Identification of Biogenic Amines and Related Compounds

Choice of the reversed-phase column packing material

Reversed phase supports from different suppliers can show large differences in chromatographic behaviour.[7] In order to find a suitable packing material for our study three different columns were tested: Two from Alltech, Adsorbosphere Catecholamine 3μm (100mm x 4.6mm I.D.) and Adsorbosphere HS C18 5μm (250mm x 4.6mm I.D.), and one from MZ-Analysentechnik GMBH, Kromasil C18 5μm (250mm x 4.6mm I.D.). DA, 5HT, and M5HT were chosen as test compounds, since DA and 5HT are the most significant amines detected in the ganglia of snails[1] and M5HT was used as an internal standard in this study.

The Kromasil column exhibited unexpected peak tailing or fronting when methanol was used as an organic modifier and only the introduction of isopropanol, instead of methanol, to the mobile phase improved the overall chromatographic performance of this column. However, the other two columns showed a good chromatographic behaviour in any case. Consequently, both of them could be chosen for further optimization study, taking into account only that the top of the shorter column filled with 3μm particles is more likely to be clogged than the longer one filled with 5μm particles, when we inject the untreated supernatant directly onto the chromatographic column in order to avoid precolumns, a possible source of band broadening. This has led us to choose the longer Adsorbosphere column in this investigation, although it results in chromatograms with excessive retention in comparison with the other Adsorbosphere column. This attempt to find the suitable chromatographic column allowed, also, the positive identification of DA and 5HT in all tissues examined. Chromatograms produced from making injections of samples onto all different chromatographic columns tested almost always showed two peaks which corresponded, in terms of individual column retention time, to DA and 5HT.

Table 1

Retention Times (min) for All Test Biogenic Amines and Related Compounds Using Different Mobile Phase Composition

		Eluent Composition		
Test Solute	**Aqueous Buffer pH = 3.3**	**As Eluent of Previous Col. plus 6% Methanol**	**As Eluent of Previous Col. plus 25mg/L SOS**	**As Eluent of Previous Col. Except for pH = 4.1**
NE	3.5	3.1	3.3	3.4
E	4.5	3.4	3.8	4.0
DOPA	6.3	4.2	4.3	4.1
DA	7.5	4.6	5.7	6.0
5HTP	21.3	9.4	10.2	9.2
3MT	23.5	9.5	13.0	13.5
5HT	24.0	10.5	14.2	15.0
DOPAC	40.2	18.9	18.2	15.7
TRYP	49.6	22.0	24.6	20.1
5HTOH	76.0	31.0	29.8	30.0
5HIAA	98.0	38.4	37.0	34.9
HVA	162.5	60.7	57.5	48.5

Effects of organic modifier

The retention times of solutes in a pure aqueous mobile phase depicted in Table 1 have also been used in solute hydrophobicity estimation, but each RPLC system provides a distinct individual hydrophobicity measure of solutes.[8,9] Generally the more polar the solute, the less it is retained and for solutes of equal molecular size and polarity, the differences in RPLC retention may arise from steric effects.[9]

As an organic solvent, such as methanol, is added to a pure aqueous eluent, the mixture becomes less and less solvophobic. At the same time its surface tension lowers. Thus, the addition of an organic modifier is expected to facilitate the transfer of polar solutes, such as the solutes under examination, from the stationary to the mobile phase.[10] In addition, the introduction of methanol to the mobile phase is accompanied by the extraction of this organic solvent by the alkyl chains of the stationary phase, which has resulted in changes in the nature of the surface of the stationary phase. Thus, the solutes will inevitably interact less with the sorbed organic modifier through dipole-dipole and hydrogen interactions.[11] As a consequence of all of the above

mentioned, the addition of methanol in the mobile phase produces a decrease in retention time for all compounds examined, but no change in the elution order of these compounds. This is depicted in Table 1 for a representative methanol concentration of 6%. Therefore, methanol can be used as a factor for shortening the run time, but not as a means of optimizing separation or identification of solutes, since the effect of methanol on the retention behaviour of solutes is, to a first approximation, independent of each individual solute.

Effects of ionic modifier

The addition of an ion-pairing agent to an aqueous mobile phase containing an organic modifier, plus a buffer to control pH, has been demonstrated to be effective in minimizing the peak tailing attributed to secondary interactions between polar ionogenic compounds and unreacted silanol groups on the silica surface and, more importantly, in controlling the retention of ionic or ionizable compound. Retention in ion-pair RPLC can be described by a dynamic ion-exchange mechanism, i.e., ion-pair formation takes place between the sample ion and hetaeron (the pairing ion) adsorbed to the stationary phase.[12-14]

In practice, the effect observed from the addition of SOS at a concentration of 25 mg/L on the retention of the examined solutes is shown in Table 1. The retention of amines, NE, E, DA, 3MT, and 5HT, and of zwitterionic solutes, DOPA, 5HTP, and TRYP, is enhanced, while that of acidic compounds, DOPAC, 5HIAA, and HVA, and of the neutral one, 5HTOH, is reduced slightly. The observed influence of SOS on the retention of these compounds is in good agreement with the predictions based on both the process governing retention in ion-pair RPLC and the degree of ionization of solutes at a given pH. At the relatively low pH = 3.3 the amines and aminoacids are protonated and thus positively charged, while the acids are essentially nonionized. On the other hand, the stationary phase should be negatively charged because of the adsorbed hydrophobic negatively charged octyl sulfonates and it should interact strongly with the opposite charged solutes causing an increase in their retention. On the contrast, the retention of unionized acids and neutrals should be almost unaffected. The observed slight decrease in the retention of these compounds can be explained by a slight lessening of their interaction with the stationary phase as it becomes more polar through adsorption of the hetaeron.

The knowledge of the effect of the ionic modifier on the retention of the solutes of interest can be used as a means of optimizing the seperation of the solutes and of further confirmation of the identities of the sample components responsible for the peaks having column retention times similar to those of

standard analytes. In other words, if we make injections of standards and samples using mobile phases containing different concentrations of SOS and the retention behaviour of the corresponding standard and sample chromatogram peaks is similar, this is a strong evidence that the same analyte is responsible for these peaks.

Effects of mobile phase pH

In RPLC the retention of ionogenic solutes, such as the compounds examined in the present study, is a strong function of the mobile phase pH with different solutes showing different behaviour both qualitatively and quantitatively.[15] As indicated in Table 1, pH affects retention in the opposite manner for acidic (DOPAC, 5HIAA, and HVA) and basic solutes (NE, E, DA, 3MT, and 5HT). In addition, the retention of the acids is more sensitive to a change in mobile phase pH than the retention of the amines, since as the mobile phase pH increases, the acids are deprotonated to a greater extent, resulting in a decrease in their retention. Conversely, the amines carry essentially the same charge over the narrow pH range examined (3.3-4.1) and their retention increases only slightly.

The retention of the neutral metabolite 5HTOH is not affected by the mobile phase pH, while the retention of the amino acids, DOPA, 5HTP, and TRYP, resembles that of the acids, i.e., their retention increases with increasing mobile phase pH. This is probably due to an initial deprotonation of the protonated amino acids to form the more polar zwitterions.

Thus, pH can be used as a paramater for optimizing separations and identifying the solutes in the samples in the same manner as the addition of SOS in the eluent.

Figure 3 gives typical chromatograms of a standard mixture and a sample from the suboesophageal ganglia as representative for the other tissues investigated. Under the chromatographic conditions of Figure 3, a complete separation of NE, DA, 5HTP, DOPAC, 3MT, 5HT, 5HTOH, M5HT, 5HIAA, and HVA was achieved and peaks corresponding to NE, DA, 5HTP, DOPAC, 5HT, and 5HIAA were identified in the snail ganglia by retention times of standards. Moreover, as systematically described above, changes in the mobile phase composition gave further evidence that these biogenic amines and related compounds were present in all snail tissues examined, except for NE which eluted close to the solvent front peak. For this reason, no clearly detectable response of NE was visible as a separate peak, especially in case where a relatively small peak with no good baseline was obtained.

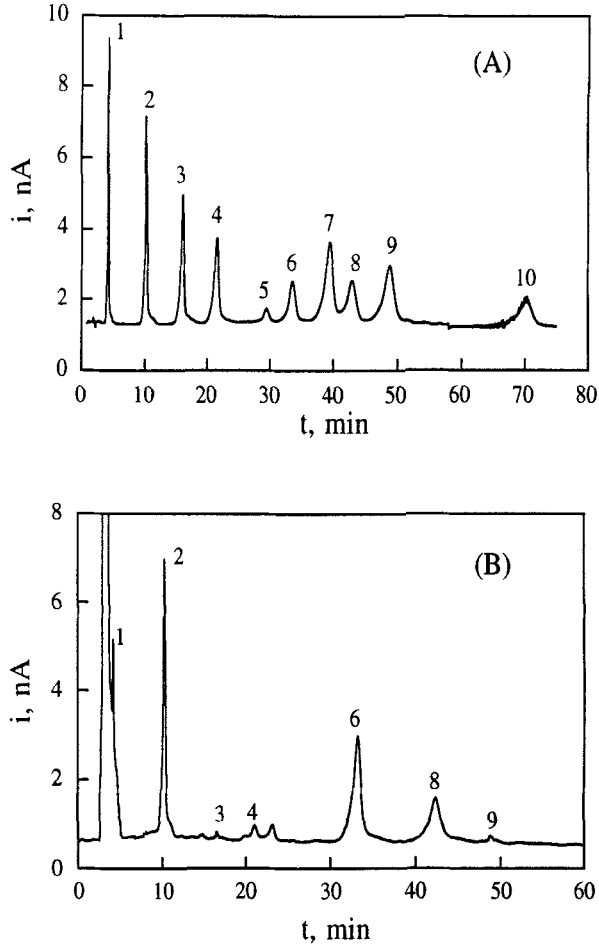

Figure 3. Typical chromatograms of 20 μL of a mixture containing 0.32 μg/mL of the reference and internal standards (A) and of a supernatant of snail ganglia (B); mobile phase aqueous buffer (pH=3.3)-methanol (96:4 v/v) containing SOS (40 mg/L). Peaks: 1=NE, 2=DA, 3=5HTP, 4=DOPAC, 5=3MT, 6=5HT, 7=5HTOH, 8=M5HT (internal standard), 9=5HIAA and 10=HVA. See text for other chromatographic conditions.

It should be noted here, that changes in chromatographic performance that occur with the continuous use of a given column, necessitate the periodic reoptimization of mobile phase parameters (SOS and methanol concentrations, and pH). However, reoptimization of column performance, using our knowledge about the effect of these parameters on retention of solutes of

interest, summarized in Table 1, can generally be accomplished in a few hours. In this investigation the necessary alterations of mobile phase composition ranged between 2-4 % for the methanol content and 20-40 mg/L for the SOS concentration.

Quantification of the Assayable Biogenic Amines and Related Compounds

Internal standardization

The use of internal standards in quantitative analytical procedures is generally considered to be superior to direct calibration. The addition of M5HT to reference solutions and tissue samples in equal concentrations, effectively controlled probable variable volumes injected and with-run variations in column or detector electrode performance. Calculation of sample concentrations was based on the comparison of the peak area ratios (relative to the internal standard, M5HT) for tissue samples to the corresponding peak area ratios for standards, i.e., $\frac{(CA/M5HT)_{sample}}{(CA/M5HT)_{standard}}$. In particular, for the quantification of a catecholamine (CA) present in a sample the following equation was used:

Concn of CA in sample, μg/g wet tissue =

$$\frac{(CA/M5HT)_{sample}}{(CA/M5HT)_{standard}} \times (\text{concn of CA in standard, } \mu g/mL) \times \frac{(\text{volume, mL})_{sample}}{(\text{weight, g})_{wet\ tissue}}$$

It is of interest to note that the use of M5HT increases the total chromatography time and, for this reason, in such assays, it is common to test internal standards, DHBA and EPI, in place of M5HT. However, the first one eluted soon after the solvent front next to DOPA and the other coeluted with DA for a given composition of the mobile phase. Consequently, since the use of M5HT simplifies establishing optimal mobile phase composition, we have chosen M5HT as an internal standard and shortened the run time for each sample using the gap between DA and 5HT for the analysis of the succeeding sample, at least in case where the most prominent biogenic amines, DA and 5HT, were only detected in tissues.

Table 2

Levels of 5HT, DA, NE, and DOPAC in Various Tissues of the Snail Helix Lucorum L.

	Concentration in μg/g Wet Tissue			
Tissue	**5HT**	**DA**	**NE**	**DOPAC**
Gaglia	15.26 ± 0.94	4.14 ± 0.75	0.51 ± 0.07	0.49 ± 0.09
Auricle	6.74 ± 0.85	0.27 ± 0.03	NA*	NA*
Ventricle	4.31 ± 0.56	0.02 ± 0.006	NA*	NA*

Values are means ± SEM (n=5)
* NA = not accurately assayable

Quantitative results

The results of the determination of the levels of biogenic amines and related compounds in different snail nervous and non nervous tissues are shown in Table 2. It is seen that 5HT occurs in greatest quantities with the most in the ganglia, followed by the auricle and the ventricle. As concerns catecholamines, DA is the dominant one in all tissues examined, although its amount in the heart muscle and especially in the ventricles is negligible compared to that in the nervous tissues. Moreover, another catecholamine, NE and the major DA metabolite, DOPAC (see Figure 3B) were found in roughly the same quantities in the ganglia but at about a 10 fold lower concentration than DA, while in heart tissues they could not be accurately measured.

In general, the results of this investigation are consistent with the results of previous studies of the gastropodes,[1,16] although there is a paucity of data detailing the quantification of biogenic amines and related compounds in the molluscan tissues.

It is worth noting that a variation in DA, 5HT, NE, and DOPAC concentrations were observed between the values determined by the analysis of different samples, see Table 2, although the samples analysed did not consist of tissues of individual animals but of two or three pooled tissues of different animals. Such variation is commonly observed in molluscan species[1,16] and is a feature of invertebrates in general.

Future Directions

In the future, it will be of interest to determine whether the biogenic amines and related compounds detected in the snail ganglia and in the heart auricles and ventricles display significant changes in their concentrations when the animals are maintained under different exogenous conditions, such as estivation, hibernation, etc. It is clearly of importance to investigate a possible correlation of differances in the levels of these edogenous compounds with behavioural dormant states of the snails.

Naturally, such studies are stongly dependent on accurate data. In this respect, we believe that the method described in this work takes into account all precautions to obtain optimum conditions and maximum sensitivity and, as a result, enables an accurate determination of catechol- and indoleamines and related compounds in biological tissues.

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