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Determination of monoamines and indoles in amniotic fluid by high-performance liquid chromatography–electrochemical detection

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SUMMARY

A technique is presented for the separation and detection in amniotic fluid of various substances associated with catecholamine metabolism. Monoamines and their metabolites were separated using reversed-phase ion-pair high-performance liquid chromatography. Detection and quantification were performed electrochemically. The retention times of 28 standards associated with the monoamines and their precursors and metabolites were evaluated with 14 different eluents. On the basis of the retention times of each standard and the modification of the retention times of the various peaks detected in amniotic fluid, the following substances were identified in this biological fluid: 4-hydroxy-3-methoxyphenylacetic acid, 5-hydroxyindoleacetic acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxy-3-methoxyphenylglycol, epinephrine, 4-hydroxy-3-methoxymandelic acid, octopamine, tyrosine and tryptophan.

INTRODUCTION

Despite the fact that it has been known for almost twenty years that monoamines are present in human amniotic fluid [1], the possible role of these substances in foetal development or during parturition has up to now received little attention. One possible reason for this lack of study is the difficulty of determining the levels of monoamines and their precursors and metabolites in amniotic fluid using the available techniques. Monoamines were first detected in amniotic fluid by Zuspan and Abbott [1] using a fluorimetric assay. Subsequently, the most commonly used methods have been thin-layer chromato-

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graphy (TLC) with subsequent quantification by radioenzymic methods and gas chromatography–mass spectrometry (GC–MS). Unfortunately, these methods suffer from a number of limitations. Radioenzymic methods are probably the most sensitive, but they do not allow the simultaneous measurement of monoamine precursors, transmitters and metabolites [2–6]. GC–MS requires expensive equipment and derivatization procedures before the analysis and is therefore time-consuming [7,8]. These techniques, then, are costly and laborious.

High-performance liquid chromatography (HPLC), especially reversed-phase ion-pair chromatography, offers important advantages over TLC and even GC [9]. Electrochemical detection provides a high degree of sensitivity for the quantification of monoamines. This papers describes a method for the evaluation of monoamines and their precursors and metabolites in amniotic fluid using reversed-phase ion-pair HPLC with electrochemical detection. The procedure is simple, cheap and sensitive and permits the simultaneous determination of 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid, HVA), 5-hydroxyindoleacetic acid (HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxy-3-methoxyphenylglycol (MOPEG), epinephrine (E), 4-hydroxy-3-methoxymandelic acid (VMA), octopamine (OCTOP), tyrosine (TYR) and tryptophan (TRP).

EXPERIMENTAL

Collection and storage of specimens

Specimens of amniotic fluid obtained at delivery were rapidly diluted (50:50, v/v) with 0.1 *M* perchloric acid containing $4 \cdot 10^{-5}$ *M* sodium metabisulphite to prevent oxidation of monoamines. The mixture was centrifuged for 15 min at 13 000 *g*, and the supernatant was aspirated and stored frozen at −70°C.

Analytical separation

An aliquot of the supernatant was injected into a stainless-steel column (300 mm × 3.9 mm I.D.) packed with μBondapak C_{18}, 10 μm particle size (Waters, Milford, MA, U.S.A.). The mobile phase consisted of $NaH_2PO_4 \cdot H_2O$ (PP), EDTA, heptanesulphonate (PIC B7) and methanol. The composition of the mobile phase was 2.56–16.56 g/l $NaH_2PO_4 \cdot H_2O$, 0.5–1.5 g/l heptanesulphonate, 0.1 m*M* EDTA and 6–12% (v/v) methanol. The pH was varied between 3.2 and 4.6 using sodium hydroxide or phosphoric acid. The mobile phase was filtered (0.45-μm Millipore filter) and the flow-rate was 1.0 ml/min using a Waters pump, Model 510.

Detection and peak identification

The electrochemical detector used was a Waters Model 460. The detector potential was varied between 0.4 and 0.9 V vs. the Ag/AgCl electrode. Using

these chromatographic conditions, fourteen different peaks were detected in the specimens of amniotic fluid. Some of these peaks were identified by modifying the various components of the mobile phase and jointly evaluating the retention time of each of the standards and of the peaks of the specimen being studied. Fourteen different eluents were used in this study. The eluent used as a base for the other thirteen had a pH of 3.6, and contained 10% methanol, 1 g/l PIC B7 and 9.66 g/l PP. We had used this eluent previously for the determination of monoamines in cerebral tissue [10]. In the first three modified eluents used only the pH was altered, the other characteristics remaining as in the base eluent. In the remaining modified eluents the pH of 3.6 was retained but the concentration of PIC B7, methanol or PP was modified. Another criterion used to identify the components of amniotic fluid was a comparison

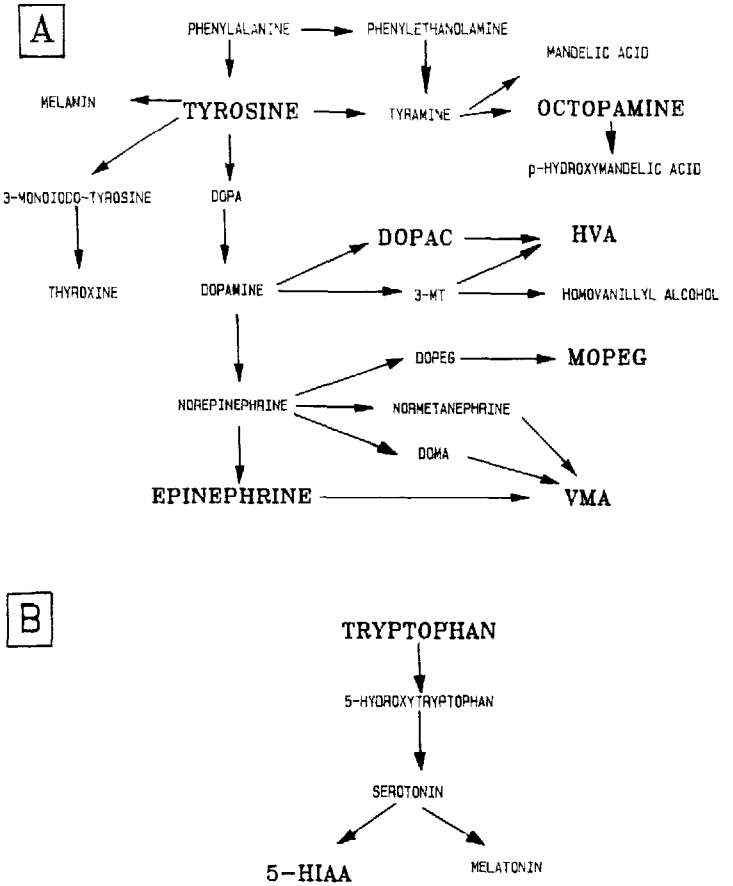

Fig. 1. Catecholamine (A) and indole (B) standards used in the present study. The substances in large script were identified in amniotic fluid; those in small script could not be detected in amniotic fluid under the chromatographic conditions used.

between the oxidation potential of each peak in the standard and those in the specimen. We considered that a peak in the specimen of amniotic fluid corresponded to a given standard if (1) modification of the retention times of a peak of amniotic fluid and of a peak of the standard was similar for all eluents used, (2) the oxidation potentials of the peak of the specimen and of the standard were similar, and (3) there were at least two conditions of the mobile phase in which the retention time of the peak of the specimen coincided with that of a single standard use in the study. The standards used (all from Sigma, St. Louis, MO, U.S.A.) included practically all the monoamines and their precursors and metabolites (Fig. 1).

RESULTS

Among the substances identified in the amniotic fluid were (1) amino acids that are precursors of neurotransmitters (tryptophan and tyrosine) (2) neurotransmitters (epinephrine) and (3) metabolites of monoamines (HVA and DOPAC from dopamine, MOPEG from norepinephrine, VMA from epinephrine and HIAA from serotonin). Octopamine was also identified.

The substances identified may be classified by retention time into substances with high retention (tryptophan, 5-HIAA and HVA), substances with medium retention (epinephrine, DOPAC and octopamine) and substances with low retention (tyrosine, VMA and MOPEG) (Fig. 2).

Modification of the pH of the eluent did not affect equally all the substances identified in amniotic fluid. Reduction of the pH increased the retention times of tryptophan, tyrosine, DOPAC, HVA, VMA and 5-HIAA, but did not affect the retention times of epinephrine, octopamine or MOPEG.

Increasing the concentration of PIC B7 increased the retention times of epinephrine, tryptophan, octopamine and, to a lesser extent, tyrosine. A reduction of retention times was observed for HVA, MOPEG and VMA. There was no significant change for 5-HIAA or DOPAC.

Increasing the concentration of PP in the eluent increased the speed of migration through the column of ephinephrine and octopamine but did not affect any of the other six substances identified in amniotic fluid. Increasing the concentration of methanol resulted in a proportional reduction in the basal retention times of all nine substances identified.

On the basis of the data obtained by injecting standards, and as a function both of the modification of retention times and of oxidation potentials, we identified nine substances in amniotic fluid. The chromatographic characteristics of these substances are shown in Fig. 2.

Even though a single chromatographic injection is sufficient to quantify most of the substances we identified in amniotic fluid, a second injection with a modified eluent is necessary in order to identify all nine substances. As can be seen from Fig. 3, using an eluent with a pH of 3.6 (10% methanol, 1.5 g/l PIC

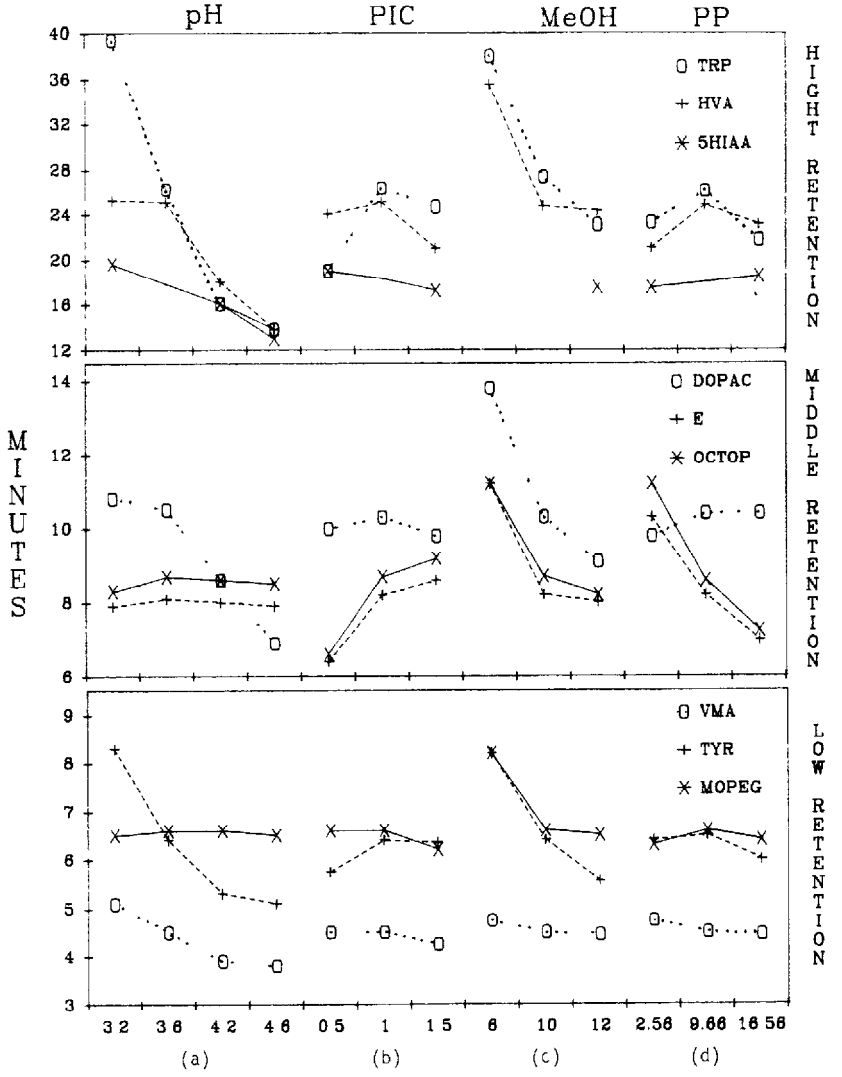

Fig. 2. Modification of retention times induced by changes in the conditions of the eluent: (a) modifications to the pH; (b) modifications in the concentration of heptanesulphonate (g/l); (c) modifications in the concentration of methanol (%); (d) modifications in the concentration of $NaH_2PO_4 \cdot H_2O$ (g/l) The substances are classified into high retention (TPR = tryptophan; 5-HIAA = 5-hydroxyindoleacetic acid; HVA = homovanillic acid), medium retention (E = epinephrine; DOPAC = 3,4-dihydroxyphenylacetic acid; OCTOP = octopamine) and low retention (TYR = tyrosine; VMA = 4-hydroxy-3-methoxymandelic acid; MOPEG = 4-hydroxy-3-methoxyphenylglycol).

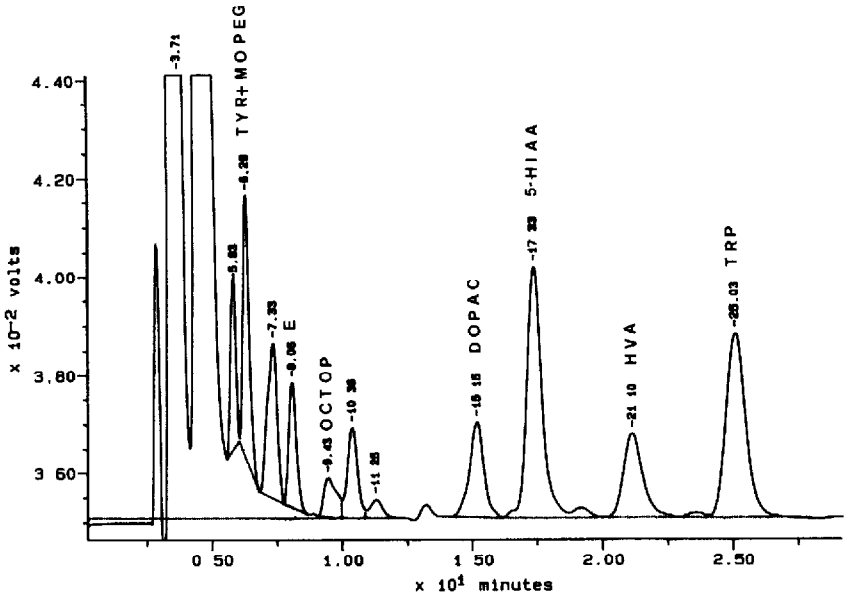

Fig. 3. Chromatogram of amniotic fluid obtained using an eluent containing 1.5 g/l PIC B7, 9.66 g/l PP and 10% methanol and with a pH of 3.6. Identifiable peaks include that of epinephrine (retention time 8.06 min), octopamine (9.43 min), DOPAC (15.15 min), 5-HIAA (17.33 min), HVA (21.03 min) and tryptophan (25.03 min). Tyrosine and MOPEG co-elute in the peak observed at 6.28 min.

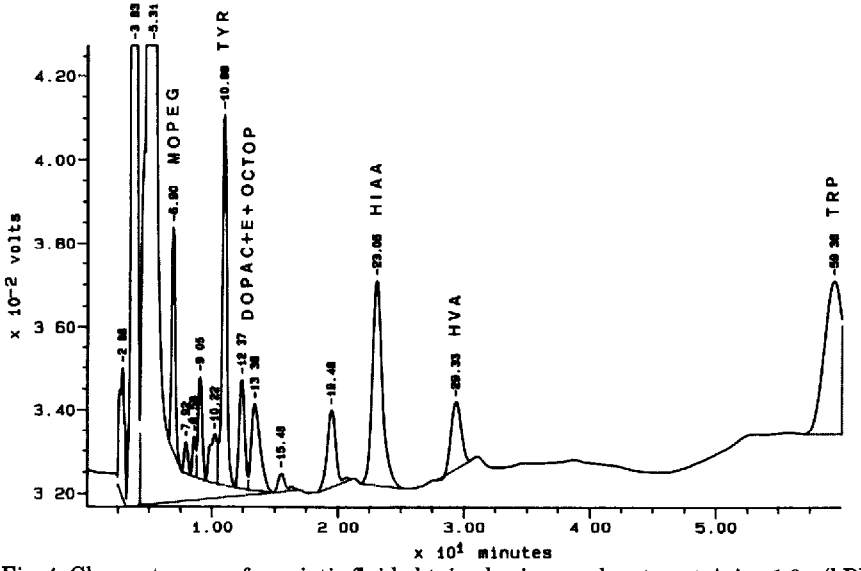

Fig. 4. Chromatogram of amniotic fluid obtained using an eluent containing 1 0 g/l PIC B7, 2.56 g/l PP and 8% methanol and with a pH of 3.0. Identifiable peaks include that of MOPEG (elution time 6.9 min), tyrosine (10.98 min), 5-HIAA (23.05 min), HVA (29.33 min) and tryptophan (59.36 min). DOPAC, epinephrine and octopamine give only the peaks observed at 12.37 and 13.38 min.

B7 and 9.66 g/l PP) it is possible to separate seven of the nine substances identified; tyrosine and MOPEG co-eluted. Fig. 4 shows how these two substances can be separated using an eluent of pH 3.0 composed of 8% methanol, 1 g/l PIC B7 and 2.56 g/l PP.

DISCUSSION

This paper describes a chromatographic technique for the separation and quantification of the amino acid precursors of monoamines (tyrosine and tryptophan) and of their principal metabolites (DOPAC, HVA, MOPEG, VMA and 5-HIAA). The main advantages of this technique are as follows: (1) it permits the simultaneous determination of the precursors and principal metabolites of the monoamines in amniotic fluid, and this determination is performed on a single specimen and with a single chromatographic injection; (2) the use of electrochemical detection results in high sensitivity; (3) it is a rapid and simple procedure, as the specimen can be prepared in 15 min and the separation and quantification of the various substances can be performed in less than 35 min; and (4) it is an economical procedure, since the products used to protect the specimen and to make the eluent are cheap. In the present study we recycled the eluent by filtering it every 48 h and were thus able to use the same eluent for a considerable number of specimens. The chromatographic column can likewise be used for a considerable number of injections (more than 500) without showing significant deterioration in retention times or other characteristics of the chromatographic peaks. As a means of protecting the column, however, we recommend that specimens of no more than 50 ml be injected and that a disposable pre-column be used.

Radioenzymic methods are probably the most used, but do not permit the simultaneous determination of catecholamines and related substances. This makes it difficult to ascertain the relative amounts of these substances in amniotic fluid. In the present study we found the concentrations of precursors and metabolites to be very much higher than those of the neurotransmitters themselves. The metabolites of dopamine detected in amniotic fluid were DOPAC and HVA, and that of norepinephrine was MOPEG. However, we cannot assert that the latter is the most important metabolite of norepinephrine in the foetus. This is because DOMA and DOPEG are not among the substances that can be evaluated using this technique since under most chromatographic conditions they elute close to the solvent front. The principal metabolite of epinephrine is VMA, a substance that can be quantified using the technique described here. However, for this purpose an eluent with a low pH (3.0) and low concentrations of PIC B7 (0.5 mg/l or less) and of PP (2.56 g/l or less) should be used in order to avoid co-elution with the solvent front. The metabolite of serotonin found in amniotic fluid is 5-HIAA. This is also the main metabolite found in neural tissue during prenatal development [11].

The numerous advantages conferred by reversed-phase ion-pair chromatography make it the method of choice for many applications. A number of models have been proposed in attempts to explain the way in which ion-pairing agents influence chromatographic separations, but the exact mechanisms remain unknown. So, it is necessary to test the action of a number of factors, such as pH, organic modifier concentration (methanol in this case) and counter-ion concentration, to induce the desirable modifications of retention times in the biological samples. We studied the four main factors that affect the retention times in this type of chromatography. The pH at which the separation is carried out is a critical factor. The dependence of the capacity ratios on pH shows on the whole the expected behaviour [12–15]. At the pH values studied here the retention of acids, such as DOPAC or HVA, decreased with increasing pH, but that of amines, such as epinephrine, was unmodified (Fig. 2). The retention times of all compounds were decreased by the addition of methanol, with the elution order unchanged and about the same selectivities. Addition of heptanesulphonate increased the retention times for amines but did not change the retention times for acids. A similar effect was detected for the addition of $NaH_2PO_4 \cdot H_2O$.

There is evidence indicating that catecholamines in amniotic fluid are derived principally from the foetal compartment [13,14]. Although the physiological role of catecholamines in the foetus and the neonate remains unclear, abnormal levels of catecholamines or their metabolites have been associated with neural tube defects [18], prenatal phaeochromocytoma [7], foetal effects of epidural maternal analgesia [19], maternal smoking [5] and intrauterine growth retardation [6]. Recently, we reported that tyrosine administration to pregnant rats induces an increase of brain norepinephrine and dopamine in the foetus [10], as well as a permanent modification of brain catecholaminergic systems during adulthood [20]. These previous data reported in laboratory animals suggest that monoamines could be neurotrophic factors in the foetus and that the ingestion of its amino acid precursors affect this prenatal role. The simple procedure reported here to evaluate, using amniotic fluid samples, the monoamines pathways during prenatal life could also be useful in the study of the physiological role of catecholamines and indoleamines in humans.

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