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Journal of Chromatography B, 769 (2002) 145–153

JOURNAL OF CHROMATOGRAPHY B

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Novel and sensitive high-performance liquid chromatographic method based on electrochemical coulometric array detection for simultaneous determination of catecholamines, kynurenine and indole derivatives of tryptophan

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Received 11 September 2001; received in revised form 17 December 2001; accepted 20 December 2001

Abstract

A novel and simple method has been developed for the simultaneous quantification of tryptophan, kynurenine and indole derivatives as well as four catecholamines, including dopamine, noradrenaline, homovanillic acid and 3,4-dihydroxyphenylacetic acid. The method utilises isocratic reversed-phase high-performance liquid chromatography with electrochemical coulometric array detection. The influence of various parameters on chromatographic performance, such as the composition and the pH of the mobile phase and the detection potentials, was investigated. Separation of 13 compounds was achieved by a mobile phase consisting of 10% methanol in 50 m*M* sodium phosphate–acetate buffer, pH 4.10, containing 0.42 m*M* octanesulphonic acid. The calibration curve was linear over the range 12 pg to 300 ng on-column. The detection limits (S/N 3) depended on the working potential and were found to be between 10 and 100 pg injected. The method was reproducible with intra-day RSDs of 0.3 to 1.5% and inter-day RSDs of 0.5 to 4%.

Keywords: Kynurenine; Tryptophan; Dopamine; Noradrenaline; Homovanillic acid; 3,4-Dihydroxyphenylacetic acid

1. Introduction

The metabolism of the amino acid tryptophan (TRP) via the kynurenine or indole pathway (Fig. 1) generates biologically active molecules that have been implicated in the pathogenesis of several neurological disorders (for reviews, see Refs. [1–3]). During the progression of neurodegenerative diseases, elevated levels of kynurenines, i.e. kynurenic acid (KYNA) [4,5] and 3-hydroxykynurenine (3HOKYN), have been observed [5–7]. Serotonin (5-HT), the indole derivative of tryptophan, and the catecholamines appear to play a role in several mental disorders, including anxiety and depression. Recent studies with animal stress models indicate that, beside the brain catecholaminergic and serotoninergic pathway, the kynurenine pathway is

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1570-0232/02/$ – see front matter
PII: S1570-0232(01)00639-0

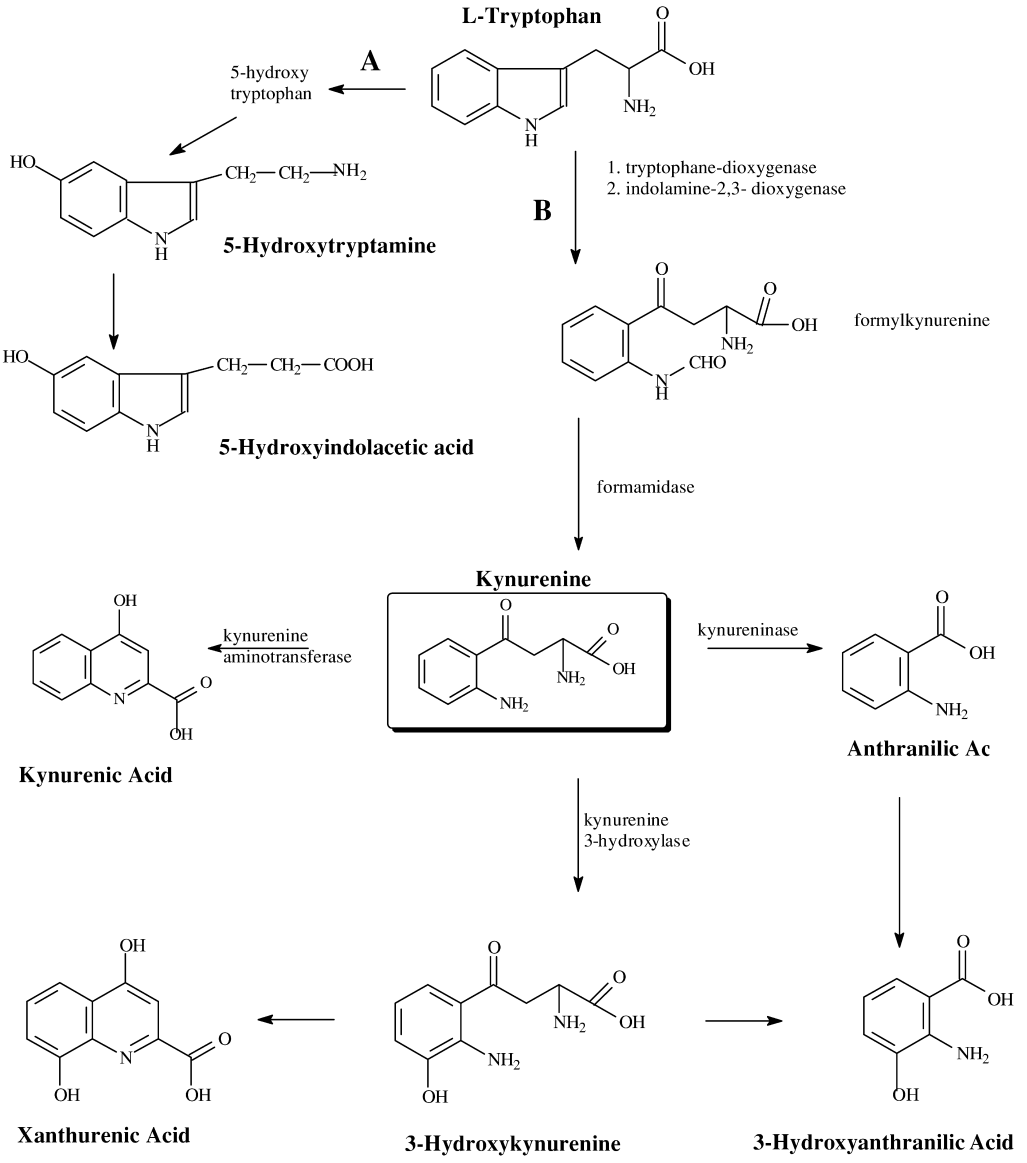

Fig. 1. The indole (A) and kynurenine (B) pathways of the metabolism of tryptophan.

also activated [8]. Because the kynurenines and serotonin have the same precursor, the pharmacological intervention or physiological changes in one pathway may influence the balance in the other. Therefore, the usage of various chromatographic group tests in the screening of metabolic disorders provides valuable and extensive information to help us understand the mechanisms of neurological diseases.

Several chromatographic protocols have been proposed to determine catecholamines [9] and individual components of tryptophan metabolism in biological fluids. Commonly used techniques are HPLC with fluorescence [10,11] or electrochemical detection [12,13] and lately HPLC with mass spectrometry [14] as well as combinations of these three techniques [14–16]. The sensitivity of HPLC with UV detection [17,18] may remain too low for biological

samples. Most of these methods are time consuming, and employ gradient elution or multi-step detection and separation systems. A frequently encountered problem during the analysis of complex samples with chromatographic techniques is the reliability of sample constituent identification. As a rule, identification is based on a comparison of the retention times of sample components and standard compounds. Reliable confirmation of peak identity generally requires additional characterisation, which may be, for example, optical or mass spectra or voltammetric responses. HPLC with electrochemical array detection uses for comparison the current/potential behaviour of standards and analytes, this extra characteristic enhancing the specificity and selectivity of the method.

The aim of our study was to develop a method to identify a large number of TRP metabolites and catecholamines in a biological sample in a single run within the shortest time possible. We combined the flexibility of reversed-phase ion-pair chromatography with the specificity of electrochemical array detection to achieve the separation of basic, acidic, and neutral compounds in the same sample.

2. Experimental

2.1. Chemicals

All standards, tryptophan, kynurenine (KYN), kynurenic acid, 3-hydroxykynurenine, anthranilic acid (ANA), 3-hydroxyanthranilic acid (3HANA), xanthurenic acid (XANT), serotonin hydrochloride, 5-hydroxyindoleacetic acid (5HIAA), dopamine (DA) hydrochloride, noradrenaline (NA) hydrochloride, homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC), and other reagents were of analytical grade and purchased from Sigma (St. Louis, MO, USA) and Aldrich (Milwaukee, WI, USA). Sodium octylsulphonate (SOS) and sodium heptylsulphonate (SHS) were from Fluka Chemie (Buchs, Switzerland). HPLC-grade methanol was obtained from Merck (Darmstadt, Germany). The ultrapure water for method development was obtained from an Aries Vaponics (Rockland, MA, USA) water purification system.

2.2. Standard and sample preparation

The primary stock standard solutions were made by dissolving a fixed amount (10–20 mg) of the component in 25 ml of 0.12 *M* perchloric acid (PCA) containing 5 m*M* sodium bisulphite. To facilitate the dissolution of kynurenine, kynurenic acid and xanthurenic acid, few drops of 1 *M* NaOH were added to the standard solutions and final dilutions were made with ultrapure water. All these concentrates were then stored in 1 ml aliquots at −80 °C and thawed prior to use at 4 °C. The stability of the concentrated stock solutions was monitored over a 6-month period and no evidence of decomposition was found. Working standards in the n*M* range were freshly prepared in 0.12 *M* PCA before each assay.

The frozen brain samples were weighed and sonicated for 30 s in 500 μl of 0.1 *M* perchloric acid containing 5 m*M* sodium bisulphite. The homogenate was then centrifuged at 13 000 *g* at 4 °C for 20 min and 20 μl of supernatant was injected into the HPLC system.

2.3. Mobile phase preparation

Stock buffer solutions of 50 m*M* monobasic sodium phosphate and 50 m*M* sodium acetate combined in a ratio of 1:1 were used as base for most mobile phase solutions. SOS in concentrations ranging from 0.42 to 1.5 m*M* and SHS in concentrations from 1 to 2 m*M* were added to influence the separation. The pH of the buffer solution was adjusted between 3.10 and 4.60 by titration with *ortho*-phosphoric acid. Methanol (6–12% v/v) was added to the mobile phase and filtered through a 0.22 μm pore size filter (GHP Polyprofilters, Gelman Laboratory) and degassed under vacuum for 20 min prior to use.

2.4. Instrumentation

The HPLC analysis was performed with an ESA Model 5600 CoulArray system (Chelmsford, MA, USA) equipped with an in-line degassing unit (Jour Research, Sweden), an ESA Model 582 pump, and an ESA Model 540 refrigerated autosampler. The detection system consisted of two coulometric array modules, each containing four electrochemical detec-

tor cells. Initially, electrode potentials were selected over the range +50 to +1000 mV with a 150 mV increment against palladium electrodes (exact potentials are given below). Chromatographic separation was achieved on an ESA MD-150 reversed-phase C_{18} column (3 μm particle size, 150×3.0 mm I.D.) with a Hypersil C_{18} pre-column (7.5×4.6 mm I.D., 5 μm). The column and detectors were housed in a thermal chamber maintained at 30 °C. A mobile phase flow-rate of 0.5 ml/min was used in all experiments. The system was controlled and the data were acquired and processed using CoulArray software (version 1.04).

3. Results and discussion

In order to achieve the separation of a 13-component mixture, the influence of various parameters (detection potentials, ion-pair reagent concentration and buffer pH) on chromatographic performance was investigated. Initial detection potentials were set from 50 mV (at electrode 1) to 1000 mV (at electrode 8) with a 150 mV increment versus the palladium reference.

3.1. Optimisation of chromatographic conditions

Preliminary experiments were performed using a mobile phase containing 0.1 m*M* monobasic sodium phosphate–acetate buffer (pH 3.10), 10% methanol and 0.55 m*M* SOS, since this solution has been used successfully for the determination of catecholamines, serotonin and 5-hydroxyindoleacetic acid from brain tissue homogenates in our laboratory [19]. In order to enhance the selectivity of this method and to additionally separate the kynurenines from the same sample, we optimised the composition of the mobile phase.

Initially, the concentration of the ion-pair reagent was varied, while other constituents of the mobile phase were held constant. An increase in the concentration of SOS from 0.4 to 1.5 m*M* or SHS from 1 to 2 m*M* predictably elevated the k' values of basic (3HOKYN, NA, KYN, DA and 5HT) or neutral (TRP) compounds (Fig. 2). The magnitude of this effect is dependent on the type of ion-pair reagent and the retention of the compound. Later-eluting basic metabolites show a greater increase in retention than earlier-eluting basic substances. On the other hand, the retention times of acidic metabolites (XANT, KYNA, DOPAC, 5HIAA, ANA and HVA)

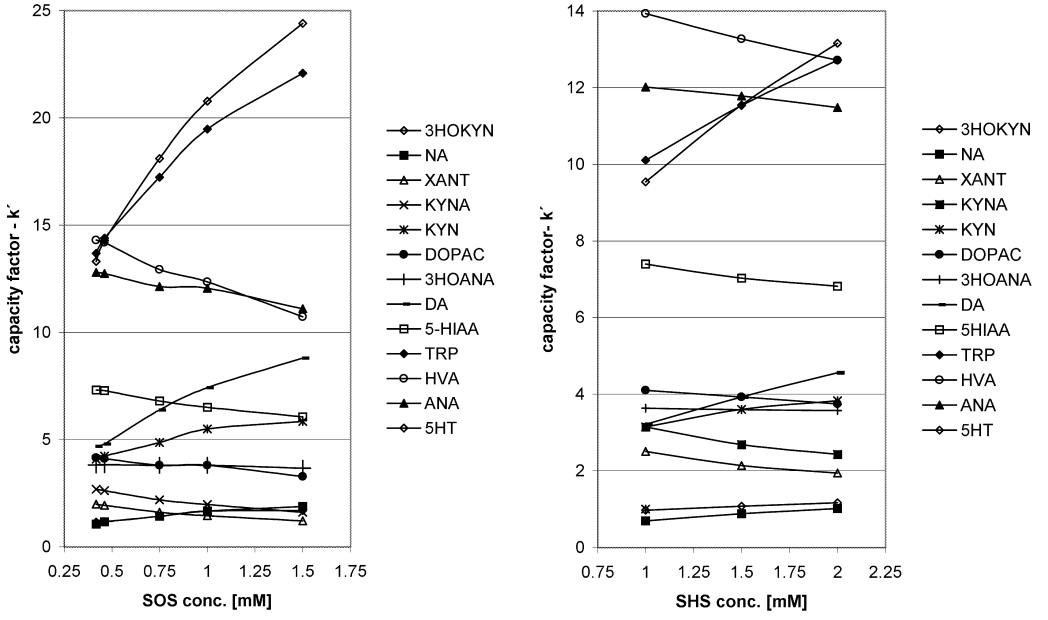

Fig. 2. Effect of the concentration of sodiumoctylsulphonate (A) and sodiumheptylsulphonate (B) on the capacity factors.

decrease slightly. Hydroxyanthranillic acid and DOPAC as well as 3HOKYN and noradrenaline exhibit very similar chromatographic behaviour under these conditions, and therefore changing either the ion-pair agent content or type in these ranges did not give a complete resolution of all 13 metabolites.

The influence of the organic modifier content on the capacity factors was predictable. An increase in the amount of methanol from 6 to 12% reduced the retention times of all components of the standard mixture, with a minor influence on the elution order (data not shown).

The effects of the mobile phase pH are related to the degree of ionisation of the sample molecules and are dependent on their pK_a values. The more-acidic carboxylic groups which are protonated at low pH will dissociate as the pH increases. Accordingly, raising the pH in the range 3.10 to 4.60 preferably reduced the retention of acidic metabolites with a carboxylic group, such as XANT, KYNA, DOPAC, 5HIAA, HVA, ANA and TRP (Fig. 3). In the pH range we used, the basic amines remain protonated and therefore their retention times change little.

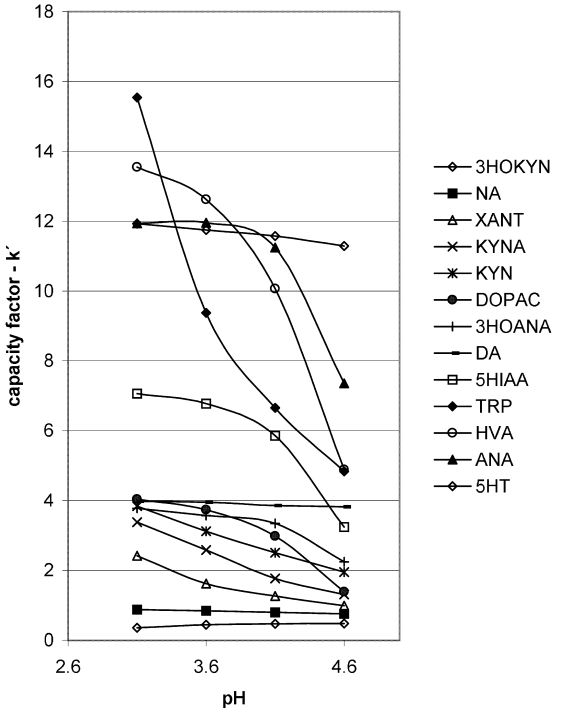

Fig. 3. Effect of mobile phase pH on capacity factors.

These results are consistent with earlier reports [20,21].

We compromised between adequate resolution and sample run time and found the following mobile phase composition to be optimal: 50 m*M* monobasic sodium phosphate, 50 m*M* sodium acetate, 0.42 m*M* sodiumoctylsulphonate, 10% (v/v) methanol, pH 4.10. As shown in Fig. 4, under these conditions all 13 substances elute from the column within 20 min.

3.2. Electrochemical behaviour

The use of a coulometric array detector for the determination of catechol- and indolamines has been reported earlier [22,23] and also the current–potential relationship of these compounds has been studied in detail [24]. Comparable information on kynurenines is far less extensive [25,26]. As catecholamines and several tryptophan metabolites contain more than one oxidisable functional group, measurable in the potential range used, we constructed oxidative hydrodynamic voltammograms (HDVs) for all analytes to increase the reliability of compound identification (Fig. 5). As can be seen from this figure, serotonin and 5-HIAA show two distinct characteristic potentials; the lower is due to oxidation of a hydroxy group and the higher is from oxidation and opening of the pyrrole ring of the indole structure. Finally, on the basis of HDVs, the cell potentials were selected so that each compound was detectable at two or three subsequent electrodes: the leading, dominant and consecutive channel. Thus, suitable cell potentials were as follows: 50 mV at electrode 1, 150 mV at electrode 2, 250 mV at electrode 3, 350 mV at electrode 4, 550 mV at electrode 5, 900 mV at electrode 6, 1000 mV at electrode 7 and electrode 8 was not used. Under the present chromatographic conditions it was possible to detect KYNA sensitively and selectively at a lower potential (1000 mV) than reported earlier (1060 mV) [26].

3.3. Assay performance

Assay linearity was examined by analysing six different levels of mixed standards in triplicate. By least-squares regression analysis, the detector response was directly proportional to the standard

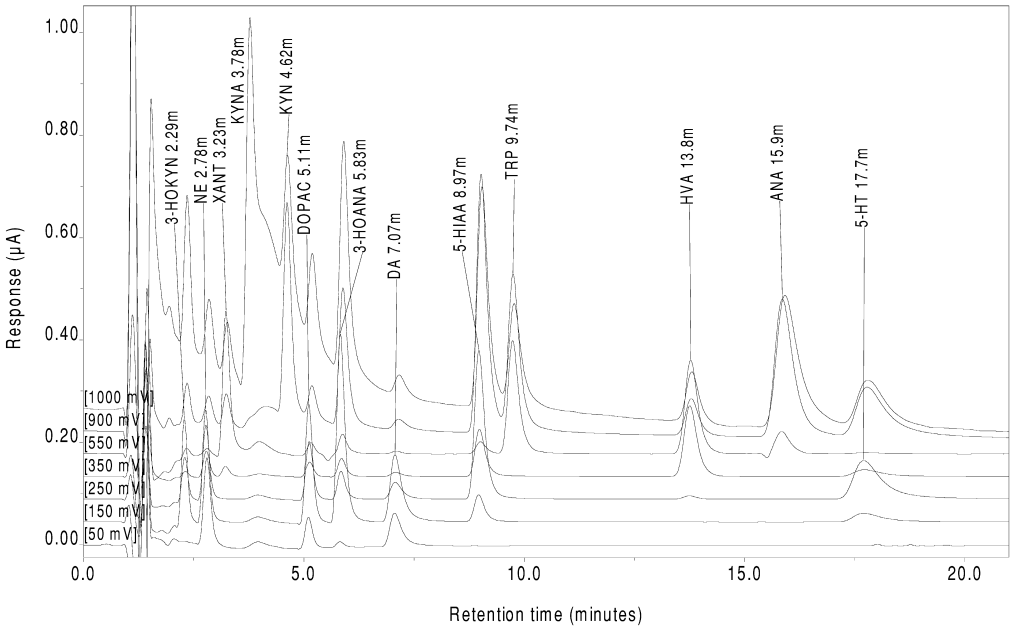

Fig. 4. Typical multichannel chromatogram of a standard mixture (60 ng ml^{-1}).

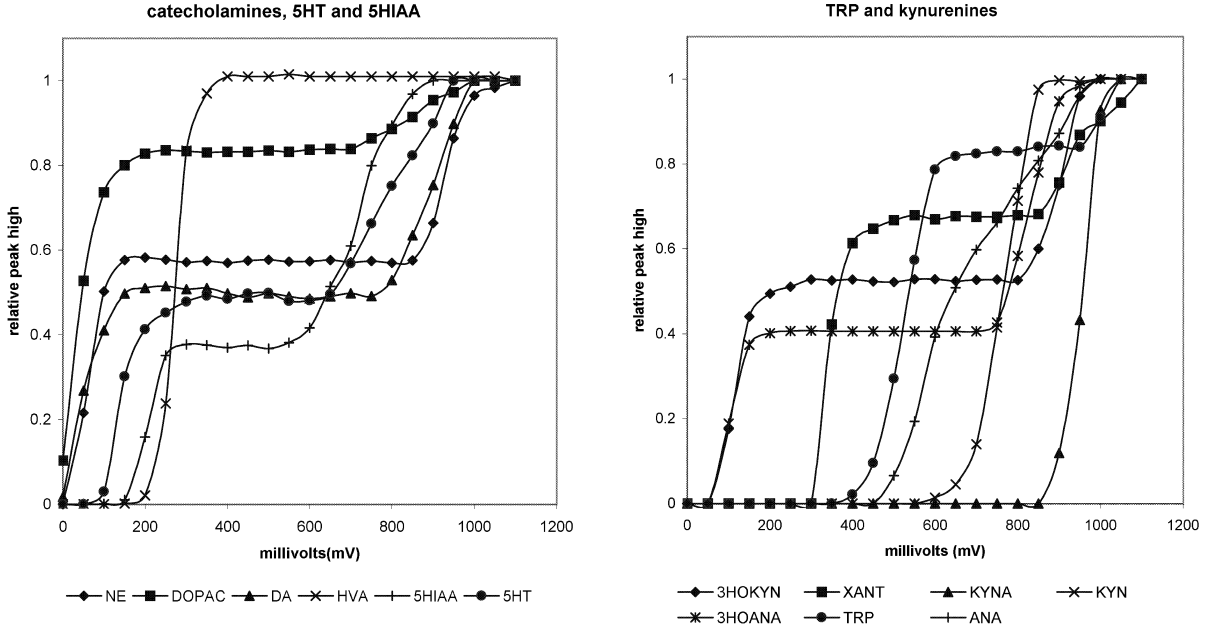

Fig. 5. Hydrodynamic voltammograms obtained by plotting relative peak heights from each standard as a function of the potential (mV) across an array of eight coulometric electrodes.

concentrations ($R^2 > 0.99$) for all substances, and calibration curves were linear from baseline values to 300 ng of each analyte injected. Data for assay performance are summarised in Table 1. The detection limits depended on the working potential and were between 12 and 104 pg per 20 μl of standard injected. Detection limits in our study (at a signal-to-noise ratio of 3), especially for kynurenines, were much lower than those published in previous reports, where they were hundreds of pg injected for electrochemical detection [11,13] or in the ng range for fluorescence or UV detection [15,18]. The repeatability was evaluated by analysing six replicates of a mixed standard. Intra-day concentration variability (RSD%) ranged from 0.3% (DOPAC) to 1.7% (anthranilic acid). Variability between runs was studied by assessing mixed standards on 10 separate days. Inter-day variability was also excellent, ranging between 0.6% (HVA) and 3.8% (kynurenine and kynurenic acid).

3.4. Determination of brain tissue samples

To demonstrate the applicability of the method, we analysed the tissue homogenate of rat brain (Fig. 6). The sample constituents were identified based on their retention times (±2%) as well as peak height ratios across the channels compared with the corresponding values of pure standards. All peaks with a ratio accuracy of >0.75 (standard and unknown peak ratios match >75%.) and a proper retention time were assessed. (The ratio accuracy is a characteristic defined by the CoulArray software which describes how well the sample response ratio between two subsequent electrodes matches the corresponding standard response ratio). Unidentified peaks of unknown origin, probably endogenous substances from brain tissue, were also detected. Analyte concentrations quantified were: NE (80.4 ng/g), XANT (227.0 ng/g), DOPAC (112.4 ng/g), DA (163.3 ng/g), 5HIAA (297.5 ng/g), TRP (70 692 ng/g), HVA (94.3 ng/g), ANA (69.6 ng/g) and 5HT (72.4 ng/g). It is not appropriate to interpret the results for a single sample as being representative of the concentrations of endogenous substances in a biological sample. A detailed discussion of the relevance of the brain concentrations of kynurenines/indoles is beyond the scope of this paper and will be presented elsewhere.

4. Conclusion

In summary, we have developed a reliable, rapid and selective chromatographic method for the simultaneous measurement of various naturally oc-

Table 1
Chromatographic and electrochemical behaviour of the 13 analytes. All compounds are listed according to their retention time

No	Compound	Abbreviation	Retention time (min)	Dominant channel potential[a] (mV)	Detection limit (pg per 20 μl injected)	Within-run RSD (%)	Between-run RSD (%)
1.	3-Hydroxykynurenine	3HOKYN	2.29	150	25	0.8	2.3
2.	Noradrenaline	NA	2.78	150	13	1.0	2.3
3.	Xanthurenic acid	XANT	3.23	550	50	1.1	0.8
4.	Kynurenic acid	KYNA	3.78	1000	104	1.5	3.8
5.	Kynurenine	KYN	4.62	1000	26	1.4	3.8
6.	DOPAC	DOPAC	5.11	150	12	0.3	2.5
7.	3-Hydroxyanthranilic acid	3HOANA	5.83	150	15	1.1	2.8
8.	Dopamine	DA	7.07	150	14	1.1	1.9
9.	Hydroxyindoleacetic acid	5HIAA	8.97	250	23	0.9	1.4
10.	Tryptophan	TRP	9.74	900	35	1.1	1.2
11.	Homovanillic acid	HVA	13.80	550	17	0.9	0.6
12.	Anthranilic acid	ANA	15.90	900	39	1.7	2.7
13.	Serotonin	5HT	17.70	250	32	1.2	1.8

[a] The dominant channel potential is the electrode potential where the maximum signal occurs for a given substance.

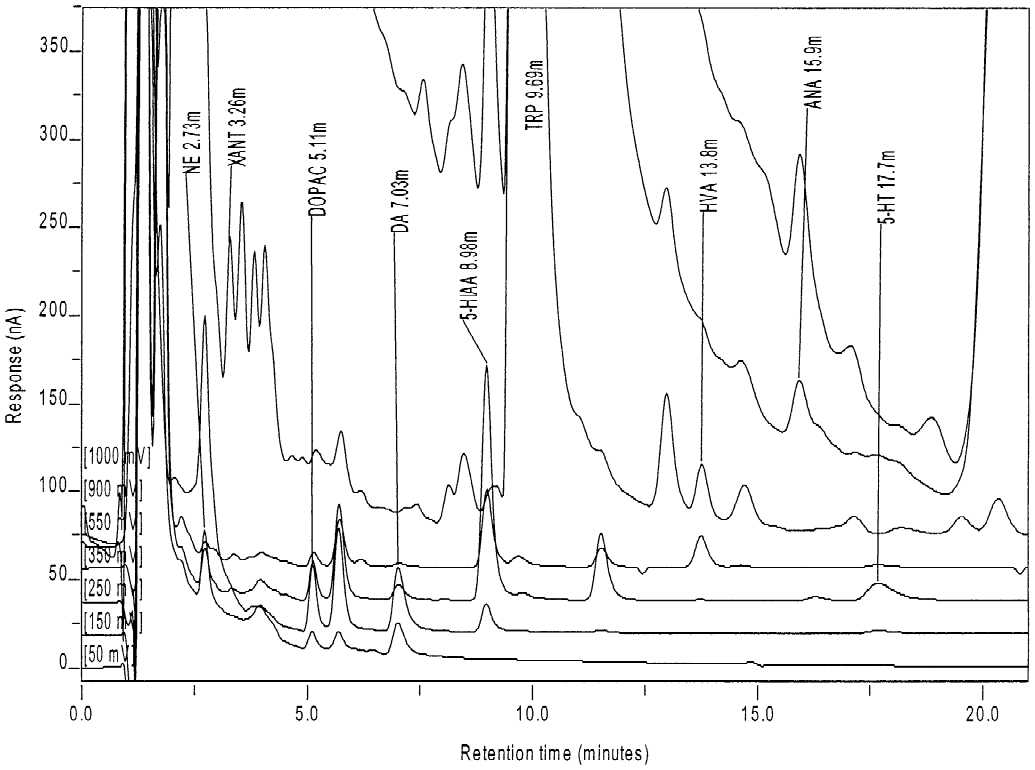

Fig. 6. Chromatogram of rat brain tissue homogenate from the striatum.

curring electrochemically active biological compounds. The electrochemical array detection mode provides additional selectivity through enhanced confirmation of the identity of sample components by their voltammetric characteristics. This method will be very useful for investigations focusing on the importance of serotoninergic, kynureninergic, and catecholaminergic systems in both experimental and clinical neuropathological studies.

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