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# Determination of neurochemicals in biological fluids by using an automated high-performance liquid chromatographic system with a coulometric array detector

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## ABSTRACT

The chromatographic separation of 33 neurochemicals was achieved by using a combined gradient of organic modifier, pH and counter-ion. A secondary separation of unresolved analytes was obtained by using electrochemical detection with a coulometric array of sixteen electrodes. The stability of the analytes was studied and data on analytical performance are reported in addition to a list of neurochemicals detected in a normal plasma sample.

## INTRODUCTION

Electrochemical detection has been used with high-performance liquid chromatography (HPLC) for more than 10 years. This technique is often used for the detection of electrochemically active neurotransmitters, and over the years many new applications have been described, covering various fields of the reversed-phase HPLC and in some instances normal-phase HPLC [1–6]. While amperometric and coulometric electrochemical techniques have been used extensively, the selectivity available with full conversion of the analyte has only been described for coulometric detectors [7].

Multiple coulometric electrodes in series can be used for characterizing molecules passing through them. This is possible by operating the cells at different potentials on the current–voltage curve of the analyte, thereby generating multiple chromatograms. From these chromatograms it is possible to obtain the ratio of the peak heights measured at different detector potentials; this ratio is typical of a pure molecule, and any deviation from this value will indicate the presence of a contaminant [7].

The ratio of the measured peaks can be obtained with a normal cell, containing two coulometric measuring electrodes in series; greater selectivity is achieved by increasing the number of electrodes in series, *e.g.*, cells containing a set of four

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coulometric electrodes potted in a single pack. Several packs of four electrodes are used in series, forming arrays of eight, twelve and sixteen electrodes [8].

As each cell in the array will react with the total amount of analyte pertinent to its potential, the concentration of the component reaching the next electrode will be decreased by the same amount. Following subsequent reactions, the component will be fully transformed when it leaves the element located in the plateau of the current–voltage curve. In a symmetric array the maximum signal will be generated by the electrode whose potential is the closest to the half-wave potential; the corresponding channel is known as the dominant channel ($T_d$). The channels immediately before and after the dominant channel are called sub-dominant channels ($T_s$).

In this way the component, will be described not only as a function of its elution time, but also as a function of its behaviour according to the potential of the reaction; this increases the selectivity of the system to help in the correct identification of the measured peaks.

In fact, the coulometric efficiency of each element of the array allows a complete separation of analytes as a function of their reaction potential, and some peaks may therefore be resolved by the detector even if they are unresolved when they leave the chromatographic column. This principle was applied to a new instrument to increase the number of species simultaneously resolved in a single analysis. By coupling the array selectivity to a gradient capability, it is possible to generate a complete picture of the components present in very complex mixtures. The first practical applications have been developed in the neurochemistry field, for different matrices, such as cerebrospinal fluid, plasma and brain tissues [9–11].

The purpose of this work was to verify the suitability of this automated system for the analysis of human plasma samples.

## EXPERIMENTAL

### *Chemicals*

The eluents used in gradient elution were purchased from ESA (Bedford, MA, U.S.A.). Mobile phase A consisted of 34.7 $\mu M$ sodium dodecyl sulphate (SDS)–0.1 $M$ monobasic sodium phosphate–50 n$M$ nitrilotriacetic acid (pH 3.35). Mobile phase B consisted of 173 $\mu M$ SDS–0.1 $M$ monobasic sodium phosphate–50 n$M$ nitrilotriacetic acid–50% aqueous methanol (pH 3.45).

### *Apparatus*

The analytical apparatus used was a Coulochem Electrode Array System (CEAS) (ESA). The instrument consists of an autosampler, capable of variable-volume injections, equipped with a 100-$\mu$l loop and a circulating glycol bath, which maintains sample vials at a temperature between 0 and 4°C prior to the injection, and two HPLC pumps capable of gradient operation from 0.05 to 10 ml/min. The output of the pumps is connected to a dynamic gradient mixer. The analytical column (80 mm × 4.6 mm I.D.) contained HR80 ($C_{18}$), 3 $\mu$m (ESA).

The detection system consisted of four cell packs in series, each containing four porous graphite working electrodes with associated palladium reference electrodes and platinum counter electrodes. The detector, column and one pulse damper were housed in a temperature-controlled compartment. Two additional pulse dampers were placed before the column and cell compartment.

The autosampler, pumps, detectors, temperature-controlled box and all associated electronic circuitry were monitored and controlled by the CEAS software, installed in a Model 286 computer equipped with a 32-megabyte hard disk and one 1.2-megabyte floppy disk drive. The computer was coupled with a high-resolution colour monitor with a "touch screen" interface and a matrix graphic printer.

This computer system performed the data storage, analysis and report generation. An appropriate software package was used for summary reports of the final data.

*Chromatographic method*

The compounds investigated were predominantly from the tyrosine, tryptophan and purine metabolic pathways and were uric acid (URIC), vanillylmandelic acid (VMA), xanthine (XANT), methionine (METH), 3,4-methoxyhydroxyphenyl glycol (MHPG), 3-(3,4-dihydroxyphenyl)alanine (DOPA), noradrenaline (NE), 3,4-dihydroxyphenylacetic acid (DOPAC), xanthurenic acid (XANTHURENIC), 4-hydroxyphenyllactic acid (4-HPLA), 3-hydroxyanthranilic acid (3-HANTR), 3-hydroxykynurenine (3-HKIN), 4-hydroxybenzoic acid (4-HBAC), *p*-tyrosine (TY), adrenaline (E), 4-hydroxyphenylacetic acid (4-HPAC), 5-hydroxyindoleacetic acid (5-HIAA), normetanephrine (NMN), homovanillic acid (HVA), kynurenine (KYN), 5-hydroxytryptophan (5-HTP), *o*-tyrosine (O-TY), dopamine (DA), metanephrine (MN), tyramine (TYR), serotonin (5-HT), 3-methoxytyramine (3-MT), melatonin (MEL), tryptophan (TP), octopamine (OCT), guanosine (GUAN), homogentisic acid (HGA) and *n*-acetylserotonin (NA-5HT).

The instrumental parameters of the separation method were as follows: cell box and column temperature, 35°C; full-scale sensitivity of all channels, 100 $\mu$A; and potentials of each of the 16 channels, starting with channel 1, – 50, 60, 120, 180, 240, 300, 360, 420, 480, 540, 660, 780, 800, 825, 850, 900 mV.

The gradient profile is shown in Fig. 1. The total analysis time was 35 min, including 5 min for stabilization between injections, at a flow-rate of 1 ml/min.

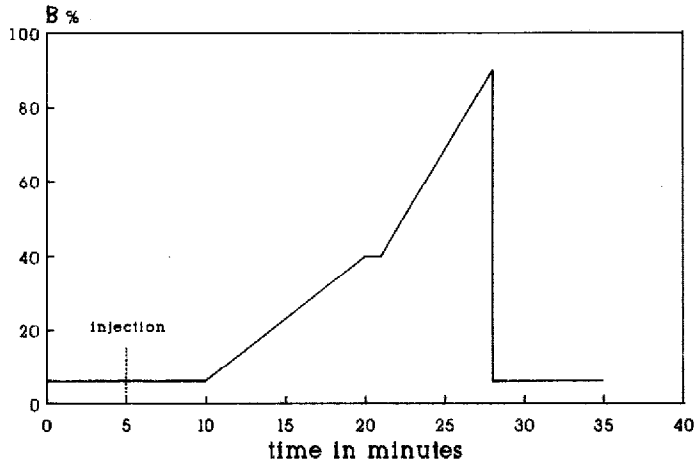


Fig. 1. Time line showing the gradient profile used in the separation.

*Peak identification and confirmation*

Peak identification was performed automatically on the basis of the traditional criterion of a retention time matching window. The peak identity confirmation was achieved by comparing the matching ratio ($R$) between a standard and the actual sample. The controlling software used a relative number to express the matching: 100% matching, $R = 1$; no matching, $R = 0$ [8].

*Standard and sample preparation*

The primary standard solutions were prepared by dissolving 10 mg of the components in a solution of 0.1 *M* hydrochloric acid–0.05% sodium metabisulphite–50% aqueous methanol. For uric acid, 0.05 *M* sodium hydroxide solution was used in place of hydrochloric acid. Individual secondary stock solutions were prepared by diluting each component with the saline solution to give a concentration of 1 μg/ml. These concentrates were subdivided into 1-ml portions. All solutions were stored at −30°C and thawed when necessary at 4°C.

A 33-component working standard was prepared by combining and diluting an aliquot of each of the concentrated mixtures (final concentration 100 ng/ml).

All plasma samples, immediately upon collection, were filtered through PM 10 membranes (Amicon, Denver, MA, U.S.A.). The filtered samples (40 μl) were injected into the CEAS.

*Precision*

To investigate the maximum analytical precision available, twenty sets of pure standards (containing 5 ng of each component) were injected and analysed under different analytical conditions.

Under strictly controlled conditions, the column compartment was thermostated at 35°C and the samples were refrigerated prior to analysis (4°C). The instrument was operated in the dark.

Under partially controlled conditions, artificial and natural light were allowed in the sample tray, the column compartment was kept at room temperature, where fluctuations of more than 5°C were observed (average value 27°C), and samples awaiting analysis were kept at room temperature.

For this quality control we tested only ten of the 33 molecules used as standards when analysing the plasma samples, *viz.*, OCT, NE, 5-HIAA, KYN, DA, MHPG, TY, 5-HT, TP and HVA.

This choice followed the criterion of evaluating a selection of molecules with different retention times, potentials and chemical characteristics representing all the other molecules considered. For all the tested substances the value of $R$ was measured.

## RESULTS

Fig. 2 shows the chromatogram of a 50-μl sample containing 5 ng each of the 33 components as external standard. The total analysis time was less than 35 min.

The reproducibility of the method was tested by repeated injections (20 times) of a standard solution containing 5 ng each of the ten substances chosen for this experiment under the conditions given under Experimental. Under the fully controlled conditions, the within-run concentration variability [relative standard deviation

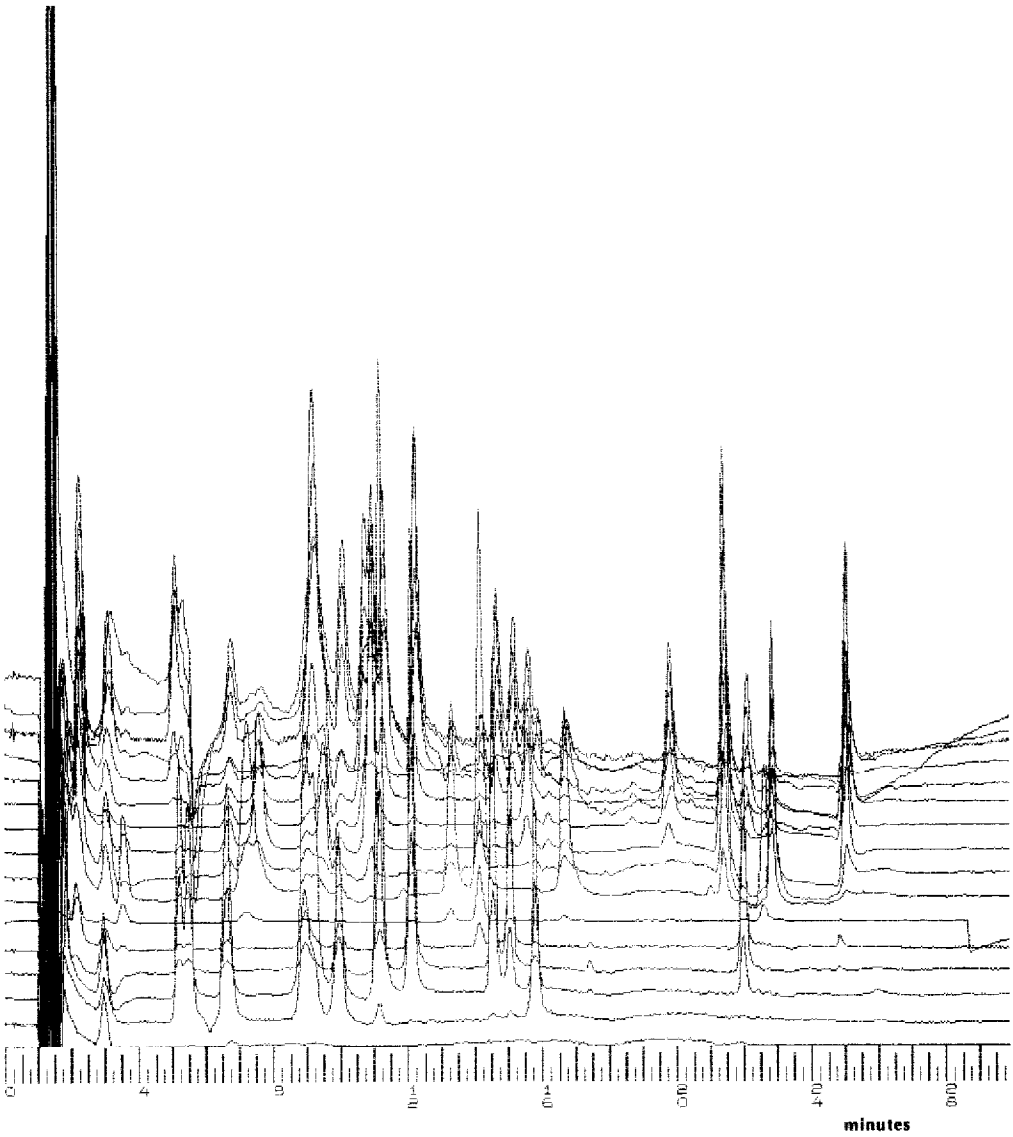


Fig. 2. Chromatogram of the 32-component standard mixture, 5 ng of each. The lines represent the signals of the sixteen electrodes. The potentials of the traces from the bottom to the top follow the values given under Experimental.

(R.S.D.)] ranged from 1 to 3% for all the substances investigated. Under the partially controlled conditions the R.S.D. was up to 10% in some instances. In particular, we observed and confirmed that 5-HIAA is degraded under the partially controlled conditions.

The results of this investigation are summarized in Table I. For each component the retention time, the standard deviation, the number of $T_d$ and of $T_s$ used for the

TABLE I

MAIN PARAMETERS FOR IDENTITY CONFIRMATION

$t_R \pm$ S.D., retention time $\pm$ standard deviation; $T_d$, dominant channel number; $T_s$, subdominant channel number; $R$, ratio matching; R.S.D. (%), within-assay precision; DL, detection limit.

| Molecule | $t_R \pm$ S.D. (min) | $T_d$ | $T_s$ | $R$ | R.S.D. (%) | DL (pg) |
|---|---|---|---|---|---|---|
| URIC | 1.73 | 4 | 3 | – | – | 4.0 |
| VMA | 2.30 | 9 | 8 | – | – | 4.0 |
| XANT | 2.34 | 14 | 13 | – | – | 20.0 |
| MHPG | 2.63 ± 0.03 | 7 | 6 | 0.97 | 2.14 | 8.0 |
| HGA | 3.42 | 1 | 2 | – | – | 4.0 |
| NE | 3.51 ± 0.02 | 2 | 1 | 0.97 | 1.35 | 6.3 |
| GUAN | 5.43 | 14 | 15 | – | – | 27.0 |
| DOPAC | 6.35 | 2 | 3 | – | – | 5.0 |
| METH | 6.35 | 15 | | – | – | 35.0 |
| DOPA | 6.35 | 3 | 4 | – | – | 7.0 |
| OCT | 6.38 ± 0.04 | 11 | 10 | 0.97 | 1.87 | 90.0 |
| 4-HPLA | 7.40 | 9 | 10 | – | – | 19.0 |
| XANTHURENIC | 7.71 | 7 | 6 | – | – | 18.0 |
| TY | 8.23 ± 0.09 | 10 | 9 | 0.96 | 1.40 | 28.9 |
| 4-HBAC | 9.31 | 12 | 11 | – | – | 25.0 |
| E | 9.87 | 2 | | – | – | 11.3 |
| 3-HANTR | 10.12 | 3 | | – | – | 13.1 |
| 3-HKYN | 10.50 | 3 | | – | – | 31.0 |
| 4-HPAC | 10.85 | 9 | 10 | – | – | 6.5 |
| 5-HIAA | 11.12 ± 0.12 | 4 | 3 | 0.93 | 3.13 | 13.0 |
| HVA | 13.40 ± 0.13 | 7 | 6 | 0.88 | 1.63 | 6.5 |
| DA | 13.73 ± 0.10 | 2 | 1 | 0.90 | 1.24 | 12.0 |
| NMN | 14.65 | 6 | 7 | – | – | 26.0 |
| KYN | 15.86 ± 0.10 | 14 | 13 | 0.45 | 2.34 | 34.5 |
| 5-HTP | 18.43 | 5 | 4 | – | – | 10.3 |
| O-TY | 18.93 | 10 | 9 | – | – | 58.0 |
| 5-HT | 19.02 ± 0.10 | 4 | 3 | 0.96 | 2.17 | 12.5 |
| MN | 20.48 | 7 | 6 | – | – | 27.0 |
| TP | 23.42 ± 0.16 | 11 | 10 | 0.97 | 5.16 | 10.4 |
| TYR | 23.59 | 11 | 10 | – | – | 59.0 |
| NA-5HT | 26.80 | 7 | 6 | – | – | 7.2 |
| 3-MT | 28.25 | 7 | 6 | – | – | 20.3 |
| MEL | 30.09 | 10 | 9 | – | – | 7.5 |

quantification, the $R$, the within-run precision (R.S.D.) and the detection limit are reported.

A chromatogram of a sample of human plasma is shown in Fig. 3, where the great complexity of the matrix can be seen. The automated data processing supplied by the CEAS allows a relatively easy collection and report of the selected analytical results.

The automatic calculation provided by the system gave the following concentrations as averages of four analyses of the same sample (40 $\mu$l), expressed in ng per injection: URIC 438.32, VMA 7.90, XANT 1295.49, MHPG 0.035, GUAN 15.21, NE

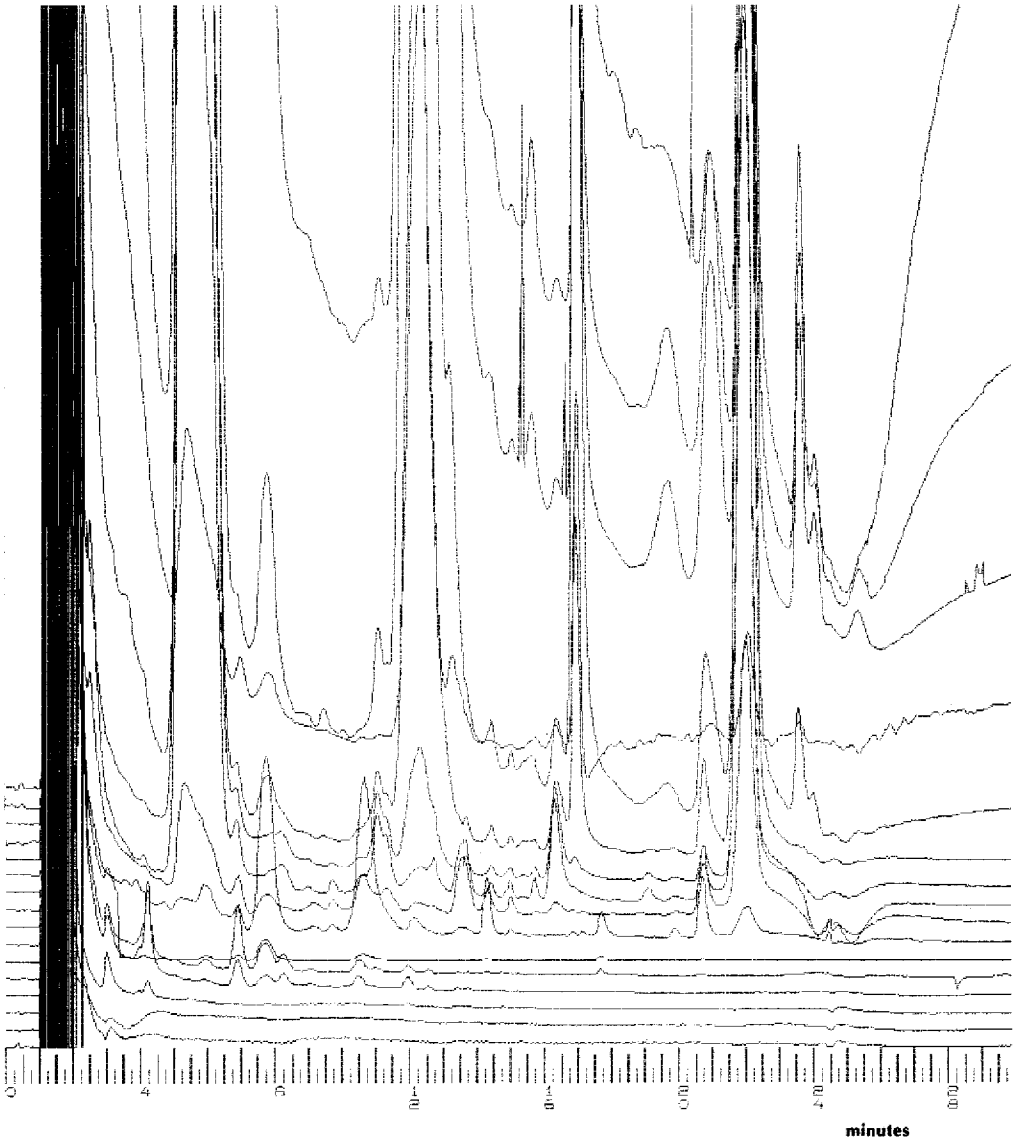


Fig. 3. Chromatogram of human plasma (40 $\mu$l), ultrafiltered with a PM 10 membrane.

0.087, METH 8.68, 4-HPLA 0.81, XANTHURENIC 0.16, OCT 0.69, 3-HANTR 0.08, 4-HPAC 4.94, 5-HIAA 0.31, NMN 0.39, HVA 0.098, O-TY 0.61, MN 0.22, KYN 133.46, NA-5HT 0.04, 5HT 0.05 and TP 53.91. Components not found in this sample were: HGA, DOPA, DOPAC, 4-HBAC, E, 3-HKYN, TY, 5-HTP, DA, TYR, 3-MT and MEL. The components not found may, of course, be present, but at concentrations lower than the detection limits indicated in Table I.

## DISCUSSION AND CONCLUSIONS

The use of the CEAS for determining neurochemicals in tissues has been reported [10,11]. In this study we have demonstrated the applicability of this technique to the analysis of biological fluids. We have presented an application to plasma, where no sample pretreatment was needed other than filtration, owing to the high selectivity of the analytical system. With reference to molecules present in very low concentrations, an enrichment step can be used as in traditional HPLC electrochemical detection, *e.g.*, alumina extraction. With the present method, we can separate 33 species in less than 35 min; in a plasma sample we were able to measure 21 of the 33 molecules searched for, and many others were detected, but not identified or quantified.

In a complex mixture such as plasma, there are several unresolved molecules, hence the retention time is not a sufficient criterion for component confirmation. Therefore, $R$ is used as a second criterion to be matched for automatic confirmation of the peaks. The closer $R$ is to unity, the better the selectivity match will be. As this is very useful information in doubtful cases, it is important to establish $R$ in an appropriate way. In order to obtain a significant $R$, two conditions must be satisfied: $T_s$ should have at least 10% of the signal measured in $T_d$, and this signal should be at least ten times greater than the detection limit. In this study, not all the molecules satisfied the first condition. This can be observed from their abnormal value for $R$ (*e.g.*, kynurenine), as the potential choice in this study was the result of the best compromise for all molecular species and gives too low a value for $T_s$.

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