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High-performance liquid chromatographic analysis of biogenic amines in cells and in culture media using on-line dialysis and trace enrichment

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Abstract

A highly sensitive method is presented for the automatic quantitative detection of DOPA metabolites in low concentrations in cells derived from the neural crest using reversed-phase HPLC in combination with fluorescence and electrochemical detection. The HPLC system was combined with on-line dialysis and on-line trace enrichment for the detection of small quantities of DOPA metabolites in culture media. Parameters like detector settings, pH, dialysis time and flow-rates are evaluated and optimized. Static–continuous dialysis can be performed at a low flow-rate concomitant with a high dialysis efficiency (up to >65%) depending on the type of DOPA metabolite. Counterflow dialysis can be used to analyse, with low efficiencies (17–29%), samples consisting of large volumes. Samples containing up to at least 7% (w/v) protein can be analysed in the low flow-rate static–continuous mode. In this last mode of dialysis, limits of detection for dopamine, norepinephrine, epinephrine and *n*-methyldopamine in DMEM/HAMF12 medium samples are 100 fmol or even lower. Serotonin is detectable at 10 fmol at a signal/noise ratio of 3. Biogenic amines were detectable at a concentration of 10 fmol/μl in a volume of 100 μl medium with an intra- and inter-assay imprecision <6.4%. This method is applied to study the differentiation level of tumour cells in culture and slices of a tumour derived from the neural crest. With this system, we also detected the excretion of DOPA metabolites from PC-12 cells after treatment with prenylamine.

Keywords: Amines; Biogenic amines

1. Introduction

Neuroblastoma and pheochromocytoma are both tumours originally derived from the neural crest. Neuroblastoma is a tumour derived from peripheral ganglia and is one of the most common extracranial solid tumours of childhood [1]. Pheochromocytoma is a tumour derived from adrenal medulla cells and is at least from a biochemical point of view more mature then neuroblastoma. One of the characteristics of cells derived from the neural crest is the

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biosynthesis of L-DOPA and its metabolites. A simplified scheme of the metabolism of L-DOPA is presented in Fig. 1. Cells derived from the neural crest are considered to be more mature in case metabolites are being biosynthesised belonging to the more proximal part of the L-DOPA metabolism such as norepinephrine. In a relatively mature tumour cell, the ratio of norepinephrine versus dopamine or L-DOPA is increased in comparison with less differentiated cells. L-DOPA and its metabolites accumulate in tumours and elevated concentrations of these compounds are found in body fluids of patients suffering from these tumours. Patients suspected of having neuroblastoma or pheochromocytoma are routinely screened for DOPA metabolites in their urine. The acid degradation products of L-DOPA and its metabolites can be increased or decreased or even be absent in these patients depending on the type and stage of the disease [2–5]. Tumour cells have been described without any metabolites of L-DOPA present. In such tumours, tyrosine-β-hydroxylase is presumably not active [2].

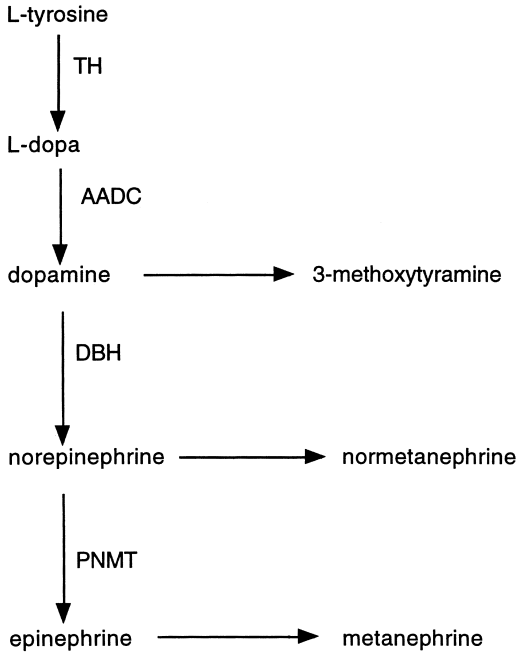

Fig. 1. Simplified scheme of the DOPA metabolism (derived from Abeling et al. [3]). Metabolites: DOPA (3,4-dihydroxy-phenylalanine), dopamine, 3-methoxytyramine, norepinephrine (or noradrenaline), epinephrine (adrenaline), normetanephrine, metanephrine. DOPA, 3-methoxytyramine, metanephrine and normetanephrine are further degraded into acid compounds. Enzymes: TH=tyrosine hydroxylase, AADC=aromatic L-amino acid decarboxylase, DBH=dopamine-β-hydroxylase, PNMT=phenylethanolamine-*N*-methyl-transferase.

To study the relationship between enzyme activity and the level of DOPA metabolites in plasma, cerebrospinal fluid and media surrounding cells in culture, several methods have been developed to measure these DOPA metabolites. Most techniques used so far are rather laborious and need an isolation step at a pH (>6) at which some DOPA metabolites are unstable [6] or need many off-line handlings like trace enrichment and removal of proteins [7–9]. Only recently a method has been described by Lenders et al. [10] using on-line trace enrichment for the detection of free metanephrines and normetanephrines in plasma by liquid chromatography (LC) combined with electrochemical detection (ED).

So far, on-line dialysis in combination with a high-performance liquid chromatography (HPLC) system has not been applied to remove protein from the sample, followed by the separation and the detection of various DOPA metabolites. During static–static dialysis, equilibrium is achieved only after many hours. To decrease the dialysis time, dialysers have been used in continuous counterflow analytical systems. However, the low dialysis efficiencies (1–10%) limit the applicability of these systems. In contrast, using static–continuous dialysis during which the recipient liquid flows continuous along the membrane whereas the donor liquid remains static in the donor chamber, efficiencies of 30–50% have been described for nitrofuran drugs. The efficiencies were significantly higher then could be achieved with continuous counterflow dialysis [11]. To analyse compounds present in low concentrations in small volumes static–continuous dialysis is preferable above continuous counterflow dialysis. In contrast, the continuous counterflow dialysis is preferred above static–continuous dialysis for the analysis of compounds present in higher concentrations in large volumes.

In this article a HPLC method is presented to analyse L-DOPA and its metabolites which were isolated at low pH from cell-line cells and neural crest derived tissues and tumours. DOPA metabolites can also be excreted by cultured cells or tissues into the surrounding medium and this excretion pattern of

DOPA metabolites reflects the pattern in the original cells or tissues [12]. For the determination of these patterns a method was developed based on HPLC which for the first time combines on-line deproteinisation (static–continuous dialysis) and on-line trace enrichment (cation-chromatography) of various DOPA metabolites including dopamine, 3-methoxytyramine, epinephrine, norepinephrine, metanephrine and normetanephrine. This method allows the quantitative determination of small amounts of these compounds and also of 5-hydroxytryptamine in culture media in a single chromatographic run. Furthermore, this method allows the comparison of the levels of the various DOPA metabolites in liquids because sample preparation and analysis are combined in one single method. Moreover, this method may also be helpful in the automated screening of agents which have differentiating effects on cancer cells derived from the neural crest.

2. Experimental

2.1. Apparatus

On-line dialysis was performed with an automatic sequential trace enrichment and dialysis module (ASTED V1.21, Gilson, Villiers, France) consisting of an autosampling injector (Model 231), two dilutors (Model 401) equipped with 1-ml syringes and poly(methyl)methacrylate flat-plate dialyser blocks with a Cuprophan membrane (15 000 Da cut-off). The donor chamber had a volume of 100 μl and the recipient chamber a volume of 175 μl. DOPA metabolites were concentrated on a cation trace-enrichment cartridge (TEC) (Supelco LC-SCX, pore diameter 100 Å, particle size 5 μm, 20 mm×4.6 mm; Supelco, Bellefonte, PA, USA). Separation of these concentrated DOPA metabolites was performed on a C_{18} column (Supelcosil LC-18-DB, particle size 3 μm, 150×4.6 mm; Supelco). The mobile phase was degassed with an on-line degassing apparatus (Gastorr, GT103, Antec, Leiden, Netherlands). DOPA metabolites were detected with a fluorescence monitor (RF-551, Shimadzu Benelux, Den Bosch, Netherlands) and/or an electrochemical detector (Decade V2.01, Antec).

2.2. Chemicals

All chemicals used were of gradient grade. The mobile phase consisted of: 2.0 m*M* sodium salt of 1-octanesulfonic acid (Fluka, Buchs, Switzerland), 94 μ*M* EDTA (Merck, Darmstadt, Germany), 0.42% (v/v) orthophosphoric acid (Merck) and 7.4% (v/v) acetonitrile (Merck). The pH was adjusted to 3.65 with 10 *M* NaOH. This mobile phase was degassed prior to use. Water used for the mobile phase was deionized.

2.3. Samples

Rat pheochromocytoma PC-12 cells and PC-12 tumours induced subcutaneously in n-Balb/c mice, were a gift from the Dutch Cancer Institute. Stock cultures of PC-12 cells were routinely maintained in 162 cm^2 plastic tissue culture flasks (Costar, Cambridge, MA, USA) in growth medium consisting of DMEM/HAMF-12 supplemented with 2 m*M* L-glutamine (Flow Laboratories, Irvine, UK), 100 I.U./ml penicillin, 100 μg/ml streptomycin (Imperial, UK), 10% (v/v) fetal calf serum (FCS) and 10% (v/v) heat-inactivated horse serum (IHS) (Gibco Laboratories, Paisley, UK). Cells were incubated in loosely capped culture flasks (Costar) at 37°C, in humidified (96%) air with 5% CO_2. Cells were passaged once a week and maintained in logarithmic growth phase. Twice a week, growth medium was refreshed. Cultures were consistently free of mycoplasma (tested with Gen-probe, ICN, UK).

Immediately after decapitation of female Wistar rats (Harlan Olac, CPB, Zeist, Netherlands) adrenal glands were collected and rapidly frozen in isopentane, cooled with liquid nitrogen, and stored under liquid nitrogen until further treatment followed. Subcutaneously injected PC-12 tumours were rapidly removed from n-Balb/c mice, frozen and stored under liquid nitrogen.

Stock solutions of L-DOPA, tyrosine, epinephrine, norepinephrine, metanephrine, dopamine, normetanephrine, *N*-methyldopamine (I.S.), tyramine, 3-methoxytyramine (not present in all standards), 5-hydroxytryptamine (these compounds were 99% pure) (Merck) were stored at 1 mg/ml doubly distilled water (pH<3) at 4°C.

2.4. Extraction and sample preparations

Adrenal medulla tissue was separated from adrenal cortex tissue at −15°C. Slices of 10 μm were prepared with a cryostat from adrenal medulla tissues and tumours and dropped in 200 μl 0.4 *M* perchloric acid for extraction of DOPA metabolites. After thoroughly mixing, the samples were incubated at 0°C for 10 min and centrifuged at 10 000 *g* at 4°C for 3 min. The supernatant was collected and the pH was adjusted to approximately 2–3 with 5 *M* K_2CO_3 and again centrifuged at 10 000 *g* at 4°C for 3 min. The last obtained supernatants were stored at −20°C until analysis of DOPA metabolites.

For cells cultured in flasks the same extraction procedure was followed with some minor modifications. Medium was carefully removed from the culture flask (immediately stored at −20°C and shielded from light). Cells were two times gently washed with ice cold NaCl/P_i (160 m*M* Na^+, 140 m*M* Cl^-, 9.4 m*M* HPO_4^{2-} at pH 7.4; without Ca^{2+} and Mg^{2+}). The cells were extracted with 200 μl of 0.4 *M* perchloric acid (with 10 fmol/μl *N*-methyldopamine as internal standard) at 4°C with intermittent scraping of the flask surface with a rubber cell-scraper (Costar). After 10 min, the suspension was transferred to a 2-ml micro test-tube (Eppendorf–Netheler–Hinz, Hamburg, Germany) and centrifuged at 10 000 *g* for 3 min. The supernatant was transferred to a 1.5-ml micro test-tube (Eppendorf–Netheler–Hinz) and the pH was adjusted to 2–3 (unless otherwise mentioned) with 5 *M* K_2CO_3. Prior to analysis with HPLC, samples were centrifuged at 10 000 *g* for 30 s. Fifty μl of the supernatant or of media were used for HPLC analysis (unless otherwise mentioned).

2.5. HPLC conditions

DOPA metabolites were measured using reversed-phase ion-pair chromatography with fluorescence (FL) detection and ED. The system set-up is described in Fig. 2. In the static–continuous mode of the ASTED, the donor-flow was stopped after filling the donor compartment of the dialysis chamber. The recipient liquid passed in counterflow along the dialysis membrane through the recipient compartment. Flow-rates of the recipient liquid were tested between 0.18–5.76 ml/min. In the continuous counterflow mode of the ASTED, the flow-rate of the donor-liquid was 1.44 ml/min whereas the flow-rate of the recipient liquid was 5.76 ml/min. The DOPA metabolites were concentrated on-line on a cation TEC. After backflush elution from this TEC, the DOPA metabolites were separated on the C_{18} reversed-phase column. For protein-free perchloric-acid extracts of cells the dialysis and trace enrichment steps were omitted. These samples were injected directly on the C_{18} column. The flow-rate of the mobile phase was 1.0 ml/min (unless otherwise mentioned). DOPA metabolites were detected with ED alone or in combination with a FL detection with

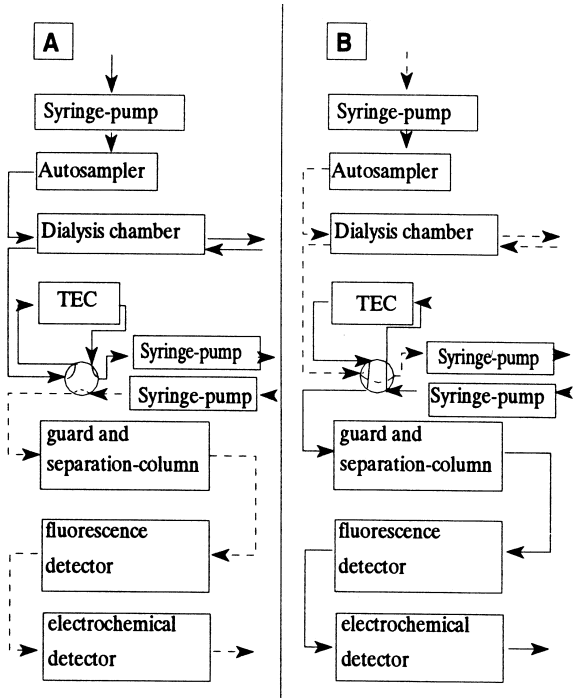

Fig. 2. Dialysis/trace-enrichment/HPLC system. Panel A represents the system during dialysis and trace enrichment on a trace-enrichment cartridge (TEC) of a sample (e.g., culture medium). Panel B is the system set-up during back-flush elution of DOPA metabolites from TEC and separation on a C_{18} reversed-phase column in combination with either a fluorescence detector (FLD) in combination with an electrochemical detector (ED) or with an ED alone. Sampling, dialysis, trace-enrichment is represented with black lines (A). In panel B, black lines represent the elution of the TEC, separation, and detection of DOPA metabolites. Dashed lines represent in both panels the flow of liquid which is not involved in the transport of DOPA metabolites.

extinction/emission wavelengths set at 280/320 nm, respectively. The electrode potential of the electrochemical detector was set at 850 mV (or 650 mV, to allow peak separation at a signal/noise ratio of 3) versus the Ag^+/AgCl electrode. During routine applications, the amplifier of the electrochemical detector was operated at a sensitivity varying from 500 pA/V to 100.0 nA/V full scale deflection (f.s.d.). At a sensitivity of 500 pA/V, a noise level of ca. 5.0% f.s.d. was observed. Chromatographic profiles were integrated using a Nelson 5.1 software package (Perkin-Elmer Nelson, Cupertina, CA, USA).

2.6. Peak identification and quantification

Peak identification was based on retention time, shape of peaks, ratio between fluorescence signal and electrochemical signal (characteristic for each DOPA metabolite at a comparable concentration as in the standards) or spiking of a sample with a known standard. The concentration of each metabolite was determined by means of an external standard calibration. *N*-Methyldopamine was used as an internal standard for the extraction procedure to correct for possible losses and dilutions during the extraction procedure. Occasionally, dopamine and metanephrine were not separated due to either degeneration of the C_{18} column or to the proportion of acetonitrile in the elution buffer.

3. Results and discussion

3.1. Detection of DOPA metabolites

To measure the various metabolites biosynthesised from DOPA in one single determination the electrode potential of the electrochemical detector was optimized via determination of the electrochemical response curves of tyrosine, L-DOPA and the various DOPA metabolites and serotonin (5-hydroxytryptamine). At an oxidation potential of 850 mV all standard DOPA metabolites of interest could be measured (the precursors tyrosine and L-DOPA were not enriched on the trace-enrichment cartridge). At a lower electrode potential (650 mV) norepinephrine, epinephrine, dopamine, *N*-methyldopamine and serotonin could be detected with a higher selectivity, while others like normetanephrine, metanephrine, tyramine and 3-methoxytyramine where almost undetectable (low electrochemical response). An oxidation potential of 650 mV was applied in those cases in which a higher selectivity was necessary at a low signal/noise ratio. In all cases, a linear relationship was observed between peak area and the amount of DOPA metabolite ranging from approximately 100 fmol (or 10 fmol for serotonin) to 100 pmol. The detection limit of the various DOPA metabolites and serotonin in the HPLC system, defined as three-times the value of the baseline noise, ranged from 10 fmol for serotonin to ca. 100 fmol for the DOPA metabolites and *N*-methyldopamine. The middle panel of Fig. 3 shows a chromatogram of a standard of relevant metabolites as detected with the ED system set at 850 mV and 100 nA/V (see below for discussion other panels).

Table 1 shows the absolute and relative retention times, electrochemical/fluorescence (ED/FL) ratios of DOPA, its metabolites and serotonin. Not only the characteristic retention times but also the ED/FL ratio (measured at a comparable concentration in standards and sample) and shape of the peaks (sharp versus broader peaks) can be used to discriminate between the various DOPA metabolites. Our lower limit of detection with the combined detection systems was limited by the fluorescence detector and was in the range of 2 pmol for most compounds. Responses of the fluorescence detector are linear over a small concentration range. Therefore, calibration at various concentrations was necessary to cover a larger concentration range.

3.2. Stability of DOPA metabolites

The stability of DOPA metabolites at different pH levels was studied in order to investigate whether biogenic amines are stable during the extraction of tumour cells derived from the neural crest with perchloric acid at low pH. We adjusted standards of DOPA metabolites, *N*-methyldopamine and 5-hydroxytryptamine (1 pmol/μl) after treatment with 0.4 *M* perchloric acid with 5 *M* K_2CO_3 to several different pH values. After centrifugation, the supernatants were directly injected onto the C_{18} column.

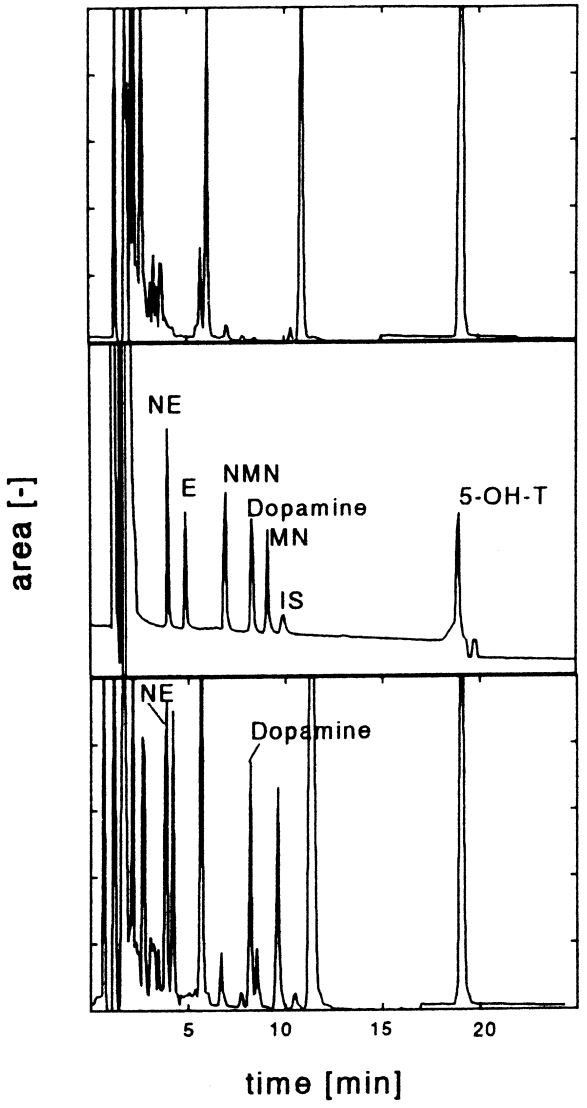

Fig. 3. Chromatographic patterns of DOPA metabolites. Upper panel: 100 μl medium in which PC-12 cells as controls were cultured. Middle panel: 100 μl of a standard of DOPA metabolites (0.1 pmol/μl) dissolved in double distilled water. Bottom panel: 100 μl medium in which PC-12 cells, treated for 5 min with 100 μ*M* prenylamine, were cultured. DOPA metabolites were detected with an electrochemical detector. The flow-rate of the mobile phase was 1.0 ml/min. NE=Norepinephrine, E=epinephrine, MN=metanephrine, NMN=normetanephrine, IS=internal standard=*N*-methyldopamine, 5-OH-T=5-hydroxytryptamine=serotonin.

Recoveries of DOPA metabolites were nearly 100% at pH values less then 4. Supernatants maintained at pH 5.0 at room temperature for 1.5 h showed recoveries of epinephrine and *N*-methyldopamine of ca. 82% and 60%, respectively. The other DOPA metabolites were nearly 100% recovered. 5-Hydroxytryptamine was hardly recovered (20%) at pH 6. This was at least partly caused by the light instability of this compound. Therefore, to prevent break-down of L-DOPA, DOPA metabolites, *N*-methyldopamine and 5-hydroxytryptamine, standards were kept at pH<3.7 and shielded from light. Under these conditions, the recoveries of all compounds were more then 97%.

Since culture media usually possess a pH of ca. 7, we tested another method to maintain stable DOPA metabolites, *N*-methyldopamine and 5-hydroxytryptamine. The addition of 3% (w/w) cysteine or 0.2% (w/w) sodium bisulfite (although toxic to cells) to culture media completely protected these compounds against oxidation at this pH.

3.3. Extraction and recovery of biogenic amines from PC-12 cells

Table 2 presents the results of the extraction of PC-12 cells with 0.4 *M* perchloric acid containing standard DOPA metabolites and 3% (v/v) cysteine. External standards were used to determine the response factors for the electrochemical detection and the fluorescence detection of the biogenic amines. Tyrosine, norepinephrine, epinephrine, normetanephrine, dopamine, metanephrine, *N*-methyldopamine and 3-methoxytyramine can be quantitatively (>90%) recovered from PC-12 cells. The recovery of tyrosine and dopamine did show a large variation due to differences in the amount of PC-12 cells present during the various extractions. These PC-12 cells already contain tyrosine and dopaniline in large quantities.

In later experiments, biogenic amines were extracted from PC-12 cells with 0.4 *M* perchloric acid in the presence of only *N*-methyldopamine as an internal standard in order to correct for possible losses and dilutions during the extraction procedure (see Fig. 3).

Table 1
The absolute and relative retention times, ED/FL ratios for various DOPA metabolites

DOPA metabolites	Retention time (min) [mean±S.D. (C.V., %)]	Relative retention time	ED/FL ratio (mean±S.D.)
L-DOPA	2.50±0.05 (1.90%)	0.14	n.d.[a]
Tyrosine	3.34±0.06 (1.80%)	0.19	n.d.[a]
Norepinephrine	4.82±0.07 (1.42%)	0.28	5.37±0.51
Epinephrine	6.72±0.12 (1.71%)	0.39	4.57±0.32
Normetanephrine	9.87±0.17 (1.75%)	0.56	2.74±0.15
Dopamine+metanephrine	14.41±0.35 (2.40%)	0.83	4.49±0.35
N-Methyldopamine (I.S.)	17.40±0.31 (1.80%)	1	7.37±0.42
Tyramine	23.87±0.43 (1.17%)	1.37	n.d.[a]
3-Methoxytyramine	31.39±0.57 (0.73%)	1.80	n.d.[a]
5-Hydroxytryptamine	37.05±0.67 (1.47%)	2.13	4.03±0.30

Values are the mean of five experiments with the HPLC column placed in the thermostatted electrochemical detector (ED) (850 mV) at 20°C and coupled with a fluorescence (FL) detector. DOPA metabolites (0.1 pmol/μl) were dissolved in double distilled water pH<3 (with HCl) and 100 μl was injected onto the column. Flow-rate of the mobile phase was 0.5 ml/min.

[a] Not detectable (n.d.) because these compounds were not detectable oxidated under these conditions. The ED/FL ratio of these compounds are not described because their amounts were below the detection level of the FL detector. Note: the ED/FL ratio varies with the concentration due to a difference in linearity between signal and concentration between the ED and the FL detector.

3.4. Cleaning procedure for the electrode of the electrochemical detector

To achieve reproducible results the pollution of the surface of the electrode, due to the relatively high electrode potential (850 mV), had to be removed on a regular basis. To circumvent laborious polishing of the electrode, we tested an alternating electrode potential varying between −1 and 1 V during 2 min which was repeated twice before each run. After this treatment, the electrode potential was set at the working electrode potential (850 mV or 650 mV).

Table 2
Recovery percentage of standard DOPA metabolites added to perchloric acid, used for the extraction of rat pheochromocytoma PC-12 cells

Metabolites	Standard (STD) (pmol)		PC-12 (pmol)		PC-12+STD (pmol)		Recovery % STD	
	Mean±S.D. (n=3)	C.V. (%)	Mean±S.D. (n=3)	C.V. (%)	Mean±S.D. (n=3)	C.V. (%)	Mean±S.D.	C.V. (%)
L-DOPA	128.7±3.0	2.3	22.5±5.2	23.0	130±2.4	1.9	81.3±4.6	5.6
Tyrosine	135.7±5.1	3.7	786.1±64.7	8.2	932±36.3	3.9	101.1±52.9	52.3
Norepinephrine	119.0±3.7	3.1	18.6±1.8	9.8	134±2.3	1.8	97.4±3.3	3.4
Epinephrine	125.3±3.1	2.5	23.8±2.6	11.1	138±2.3	1.7	92.5±3.3	3.6
Normetanephrine	118.3±3.4	2.8	n.d.		109±2.3	2.1	92.1±4.1	4.5
Dopamine	122.7±2.3	1.9	834.8±21.2	2.5	956±23.6	2.5	99.8±22.5	22.9
Metanephrine	118.3±4.3	3.6	n.d.		114±1.7	1.5	96.4±4.6	4.7
N-Methyldopamine (I.S.)	126.3±3.4	2.7	n.d.		117±1.8	1.5	92.6±3.8	4.1
Tyramine	135.7±3.8	2.8	n.d.		106±2.3	2.2	78.1±4.4	5.6
3-Methoxytyramine	122.7±2.5	2.1	n.d.		111±2.3	2.1	90.4±2.7	3.0
5-Hydroxytxyptamine	98.0±6.9	7.1	n.d.		48±12.9	26.8	48.9±14.7	30.0

Perchloric acid (0.4 *M*) consisted of standard DOPA metabolites (100 pmol) and cysteine (3%, v/v). Ca. $1.4 \cdot 10^6$ cells were used (n=3). DOPA metabolites were injected on the HPLC column without dialysis and trace-enrichment and were quantified by fluorescence detection.

Stabilisation of the electrode potential needed generally approximately 30 min, after which the change of the baseline was less than 500 pA/V. During this period, dialysis of the succeeding sample was already started. With this procedure it was not necessary to open the electrochemical detector cell for cleaning the electrode. The electrochemical detector cell is now only routinely opened for refilling the reference electrode. Without this cleaning procedure, a large inter- (up to 30%) and intra-day coefficient of variation for the analyses of biogenic amines without adjustment of the results by means of the internal standard was observed. This cleaning procedure allows us to analyse biogenic amines quantitatively in many samples with low intra- and inter-day coefficient of variation (C.V.) [HPLC in combination with dialysis and trace enrichment; intra- ($n=4$) and inter-day ($n=7$) imprecision <6.4%, see Section 3.7].

3.5. On-line trace enrichment

The on-line trace enrichment is only relevant for the detection of biogenic amines present in culture media, plasma and cerebrospinal fluid since the concentration of these compounds is generally to low to detect with conventional methods in contrast to the concentration of these compounds in urine. Different concentrations of standards of L-DOPA, DOPA metabolites, *N*-methyldopamine and 5-hydroxytryptamine in doubly distilled water (pH<3) were loaded on the TEC and were eluted in back-flush. Separation of DOPA metabolites was performed on a reversed-phase C_{18} column. Recoveries after trace enrichment versus direct injection on the C_{18} column of 100 pmol norepinephrine, epinephrine, normetanephrine metanephrine, dopamine, tyramine, and 5-hydroxytryptamine, were approximately 90% with intra-assay C.V.<5% with the ED system set at 850 mV and 50 nA/V. L-DOPA and tyrosine had almost no retention on this column. The recovery of 3-methoxytyramine was 77±7% (mean±S.D., $n=3$) at a load of 100 pmol. At least 250 pmol could be loaded on this trace enrichment cartridge (results not shown).

Different lengths of the trace-enrichment column (10 and 20 mm×2 mm) were tested. In the ASTED system a syringe pump is used to bring the dialysate onto the trace-enrichment column. This syringe pump can only be operated with trace-enrichment columns having a low back-pressure (<1.2 p.s.i.; 1 p.s.i.=6894.76 Pa). The 20 mm trace-enrichment column showed too high back-pressure to be operated in the ASTED system. Therefore, in the final set-up only a 10×2 mm TEC was used.

3.6. Dialysis

The influence of the flow-rate of the recipient liquid in the dialysis cell was studied during static–continuous (donor/recipient) dialysis (Fig. 2A and B). The time during which dialysis was performed was inversely related to the flow-rate of the recipient liquid in such a way that a constant volume of the recipient liquid was used. Increasing the flow-rate of the recipient liquid under these conditions did not improve the mass transfer (results not shown). This is probably because the time available for dialysis becomes a limiting factor. Although the mass transfer per unit of time might have increased (a steeper average concentration gradient of DOPA metabolites), the total time available for dialysis decreases with increasing flow-rate of the recipient liquid and a constant recipient liquid volume.

The time for static–continuous dialysis was kept constant at 5.5 min, while the volumes together with the flow-rate of the recipient liquid were varied. The highest efficiency was noticed at the lowest flow-rate (0.18 ml/min) tested. This result is in line with those obtained for other compounds [11]. The recovery of biogenic amines (100 μl, 100 fmol/μl) in these experiments at the lowest flow-rate varied from 35±4% (mean±S.D.; $n=3$) for 3-methoxytyramine to 65±5% (mean±S.D.; $n=3$) for norepinephrine depending on the type of DOPA metabolite. Equilibrium dialysis (static–static dialysis) would be the best option, however this would consume too much time. Therefore, further experiments were performed at the lowest flow-rate (0.18 ml/μl). A larger volume of the recipient liquid did improve the recovery. However the break-through volume of the TEC (ca. 15 ml) limited the recipient volume used during dialysis.

No detectable influence of the amount of human serum albumin (up to at least 7%, w/w) was noticed on the efficiency of dialysis of 1 pmol/μl standard

DOPA metabolites in acidic double distilled water. Therefore, the amount of protein present in culture media is not expected to limit the dialysis of DOPA metabolites from these culture media.

3.7. Overall limits of detection, recovery, reproducibility using on-line dialysis and trace enrichment in combination with HPLC for the detection of biogenic amines in liquids

In order to establish the overall limits of detection of DOPA metabolites present in liquids containing proteins with our HPLC system we spiked DMEM/HAMF-12 culture medium containing 10% (v/v) fetal calf serum (with 3% cysteine) with small amounts of DOPA metabolites. Based on the ED profiles the limits of detection, defined as three-times the value of the baseline noise, proved to be at least 100 fmol for dopamine, norepinephrine, epinephrine and *N*-methyldopamine (ED set at 650 mV), normetanephrine and metanephrine (850 mV) at a sample volume of 100 μl. The recovery was linear up to at least 10 pmol. An even lower detection limit of 10 fmol was observed for serotonin (850 mV). The limits of detection of concentrations of DOPA metabolites, *N*-methyldopamine and 5-hydroxytryptamine are depending on the volume of the sample used for dialysis which may vary from 100 μl up to 5000 μl in the static-continuous dialysis. The recovery obtained with 5000 μl sample volume is limited by the maximum volume of the recipient liquid with respect to the breakthrough volume (10 ml) of the TEC.

The overall recovery of 100 fmol/μl final concentration of DOPA metabolites added to 100 μl DMEM/HAMF 12 medium in the presence of 10% fetal calf serum and 10% inactivated horse serum in comparison with DOPA metabolites directly separated on the reversed-phase column was dependent on the type of metabolite (up to >65%). Moreover, the same overall recovery compared with DOPA metabolites in acidic doubly distilled water (pH<3) after dialysis, trace enrichment and separation varied from 98–100% with an intra-assay imprecision <4.2% ($n=4$) and an inter-day imprecision <6.4% ($n=7$). This result makes it possible to measure DOPA metabolites by using external standards injected directly on the C_{18} column to determine response factors of the DOPA metabolites for ED and FL detection and analyse the results from samples by comparison with external standards which underwent also dialysis and trace enrichment.

In the continuous counterflow dialysis a sample volume of 2500 μl was easily analysed. In the counterflow dialysis mode of the ASTED (flow-rate donor liquid is 1.44 ml/min, flow-rate recipient liquid is 5.76 ml/min) with a donor-liquid volume of 300 μl a maximum recovery varying from 17.3±1.6% (mean±S.D., $n=6$; 3-methoxytyramine) to 29.0±5.6% (mean±S.D., $n=6$; 5-hydroxytryptamine) was obtained with a mixture consisting of DOPA metabolites and 5-hydroxytryptamine.

3.8. Examples of DOPA metabolites extracted and excreted from cells

The most abundant compound in perchloric acid extracts from PC-12 cells was tyrosine (component of culturing medium). Furthermore, small amounts of L-DOPA, norepinephrine and epinephrine and a rather large amount of dopamine were present. We compared the DOPA-metabolite levels in PC-12 cells with levels observed in cryotome slices of PC-12 tumours induced subcutaneously in n-Balb/c mice and with DOPA-metabolite levels in freshly isolated rat adrenal medullae. To exclude variations induced by different extraction procedures (single cell layer versus tissue layer), ratios between several DOPA metabolites are preferred instead of absolute values (Table 3). In the established PC-12 cell line the norepinephrine/epinephrine ratio (0.7±0.2; mean±S.D., $n=3$) was approximately equal and the dopamine/norepinephrine ratio was extremely high compared to that observed for PC-12 tumours induced in mice. In these tumours, norepinephrine was considerable increased whereas epinephrine was almost undetectable. Therefore, the norepinephrine/epinephrine ratio in these PC-12 tumours was ca. twenty-fold increased in comparison with PC-12 cells cultured in vitro. The dopamine/norepinephrine ratio in these tumours was decreased compared to that observed in PC-12 cells. In adrenal medullae from eight-month-old Wistar rats, the norepinephrine/epinephrine ratio was again almost equal and slightly lower as observed in the PC-12 cells cultured in vitro. The dopamine/norepinephrine ratio of these

Table 3
DOPA metabolite ratios in cultured rat pheochromocytoma PC-12 cells, in PC-12 tumors sc. induced in n-Balb/c mice and in adrenal medullae from healthy female Wistar rats

	Dopamine/norepinephrine	Norepinephrine/epinephrine
PC-12 cells in culture	4600±600 (x±SD, n=3)	0.7±0.2 (x±SD, n=3)
PC-12 tumor in n-Balb/c mice	2–3 (n=2)	21 (n=1)
Adrenal medulla: Wistar rat 8 months old	0.04±0.02 (x±SD, n=3)	0.2±0.02 (x±SD, n=3)

PC-12 cells were cultured in DMEM/HAMF12 with 10% fetal calf serum and 10% inactivated horse serum.

adrenal medullae was very small compared to these PC-12 cells. Apparently, in PC-12 cells the activity of dopamine-β-hydroxylase was very low causing elevated levels of dopamine. However, in adrenal medulla tyrosine-hydroxylase, dopamine-β-hydroxylase, PNMT, all enzymes involved in the DOPA metabolism seemed to be quite active, causing no substantial elevated concentrations of one of the intermediate DOPA metabolites. The difference between the ratio's observed in tumours and in the cultured PC-12 cells might have been induced by the presence of differentiation inducing agents in the mice, such as nerve growth factor which is known to be able to influence the expression of the gene encoding dopamine-β-hydroxylase.

In order to be able to monitor the biochemical differentiation level of cultured tumour cells derived from the neural crest, the concentrations of the various DOPA metabolites belonging to the more proximal part of the DOPA metabolism in the cells (and in the surrounding culture media due to ''leaking'' of DOPA metabolites from cells) must be quantified. Moreover, PC-12 cells can be forced to excrete DOPA metabolites into the medium which can subsequently be analysed by HPLC without destroying the cells. As an example, PC-12 cells were treated with 100 μ*M* prenylamine during 5 min. Analysis of 100 μl DMEM/HAMF12 medium via static–continuous (0.18 ml/min) dialysis and trace-enrichment coupled with HPLC revealed a quantity of approximately 37 pmol norepinephrine/10^6 cells and 35 pmol dopamine/10^6 cells (Fig. 3). These values are corrected for the recoveries from standards which underwent also dialysis and trace-enrichment. A number of peaks not present in the standard (middle panel) were present in the medium prior to the treatment (upper panel). Other peaks appeared just after the treatment (see bottom panel).

4. Conclusions

The on-line combination of dialysis and trace enrichment with HPLC is an elegant technique for the fully automated removal of protein and the detection of trace amounts of DOPA metabolites in liquids. During static–continuous dialysis with the ASTED at the lowest flow-rate for recipient liquid, a high recovery of DOPA metabolites can be obtained from a sample with a small volume. A low recovery (counterflow dialysis mode of ASTED) can be sufficient for samples of large volumes which reduces the time needed for analysis. In general, ED can be operated at 850 mV with an automatic cleaning procedure before each analysis.

Detection of DOPA metabolites in cells derived from the neural crest cultured in vitro and in culture media might be useful for monitoring in vitro effects of agents such as analogs of nucleotides on differentiation of pheochromocytoma and or neuroblastoma cells. For this purpose mainly antibodies directed to differentiation markers, like dopamine-β-hydroxylase, are in use at this moment. The ratios between amounts of various succeeding metabolites in the metabolic pathway of L-DOPA and the absolute quantity of DOPA metabolites are preferred parameters since the presence of enzymes in cells does not always reflect the concentration of DOPA metabolites in body fluids and the physiological effect of these compounds in vivo [2,3,12]. Although techniques were already available that can detect low quantities of some specific DOPA metabolites, most

of these techniques suffered from lack of information concerning the other DOPA metabolites and/or from laborious off-line handlings and/or low sensitivity.

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