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Method for simultaneous measurement of norepinephrine, 3-methoxy-4-hydroxyphenylglycol and 3,4-dihydroxyphenylglycol by liquid chromatography with electrochemical detection: application in rat cerebral cortex and plasma after lithium chloride treatment

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Abstract

An assay was developed to quantify norepinephrine (NE) and its metabolites (MHPG and DHPG) by high-performance liquid chromatography with electrochemical detection method (HPLC-ECD) in brain tissue and plasma of rats treated by LiCl. Separation on C_{18} column was obtained by a mobile phase consisting of 4.5% methanol in buffer (0.1 M sodium acetate, 0.2 M citric acid) containing 0.2 mM ethylenediaminetetraacetic acid disodium salt (EDTA Na_2) and 0.4 mM sodium octylsulfate, operated at a flow rate of 0.8 ml/min. A potential of +0.78 V was applied across the working and reference electrodes of the detector. The precision was in the range 2.88–4.35% for NE, 5.94–11.0% for MHPG and 1.97–4.40% for DHPG. Accuracy was 98.8–99.3% for NE, 97.4–100% for MHPG and 96.1–101% for DHPG. The limit of detection was 0.6 ng/ml for NE, 0.5 ng/ml for MHPG and 0.2 ng/ml for DHPG. The linearity is over the range 20–60 ng/ml for NE, 7–23 ng/ml for MHPG and 6–20 ng/ml for DHPG. The assay has been applied successfully to measure simultaneously cortex and plasmas concentrations of these three catecholamines in rats.

Keywords: Norepinephrine; 3-Methoxy-4-hydroxyphenylglycol; 3,4-Dihydroxyphenylglycol; Lithium chloride

1. Introduction

Norepinephrine (NE) as other monoamines play an important role as neurotransmitter in the central nervous system. 3-Methoxy-4-hydroxyphenylglycol (MHPG) is one of its metabolites. A correlation between levels of MHPG in cerebrospinal fluid and plasma in drug-free affective disorder patients has been reported [1]. It has been clearly demonstrated that lithium was an effective treatment of manic-depressive illness, although its mechanisms of action remain unclear [2,3].

Sopranzi [4], Gross and Coll [5] and Schildkraut and Coll [6] have studied the effect of lithium on the release of norepinephrine in slices of cerebral cortex and plasma from rats.

Several methods for brain and plasma norepinephrine measurements have already been described. High-performance liquid chromatography (HPLC) is the most used method for NE determination. Several authors have described methods for simultaneous measurement of NE and DHPG [7–19] or NE and MHPG [20]. But, there are only few recent articles with simultaneous determination of NE, MHPG and DHPG [21–23]. Described methods are based on ion exchange separation. The conditions have been optimised for using electrochemical detection (ECD). The method can use only a glassy carbon cell and a silver/silver chloride reference electrode [24,25] or a dual-electrode detector with two cells placed in series [26,27]. In this case, NE was oxidized at the upstream electrode and the oxidation product detected at the downstream electrode to increase selectivity. Generally, the stationary phase consisted of a reverse phase column C_{18} [26,28–31]. More recent methods used two serially connected C_{18} columns [25,29] with

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improved separations. For the mobile phase, pH is always acid (pH 2.5–5.0).

Different authors used either 3,4-dihydroxybenzylamine (DHBA) [18,25,32], or deoxyepinephrine [31] or isoproterenol [29] as internal standard (IS) to confirm accuracy. Others determine the compound concentrations by chromatographic peak areas using the external standard method [26,30]. Simultaneous determination of MHPG, DHPG and NE was previously assayed using liquid chromatography with electrochemical detection [23]. But this method required pretreatment by adsorption onto aluminia.

We developed a HPLC with electrochemical detection method to measure the variations of NE, MHPG and DHPG in rat brain and plasma. Our method can detect either NE or its common metabolites MHPG and DHPG simultaneously without pretreatment, in only one injection and then offer a more accurate way of measuring NE turnover.

2. Experimental

2.1. Reagent

DL-3,4-Dihydroxyphenylglycol (DHPG), (−)-arterenol (+) bitartrate salt monohydrate (norepinephrine), 4-hydroxy-3-methoxyphenyl glycol hemipiperazinium salt (MHPG), 3,4-dihydroxybenzylamine hydrobromide (DHBA), sodium octyl sulfate (OSS), were supplied by Sigma (St. Quentin-Fallavier, France), sodium acetate, citric acid, methanol, ethylenediaminetetraacetic acid disodium salt (EDTA Na_2), potassium hydrogenocarbonate, hexane, sulfosalicylic acid, ethylacetate, sodium chloride by Carlo Erba (Rueil Malmaison, France).

All chemicals used in this study were of analytical-reagent grade.

2.2. Animals

In all experiments, female WISTAR rats (180–220 g b.w.) were used. They were kept under a standard 12:12 h light-darkness cycle and were sacrificed at different time during the day. Groups of 6–10 rats were subjected to intraperitoneal injections (IP) with the following dosing regimens:

- single injection of lithium chloride (2 mmol/kg/IP) was performed at 8:30 h and animals were killed at the following times: 1.5, 3, 6 and 24 h after injection,
- single injection of sodium chloride (2 mmol/kg/IP) was performed in the same conditions with sacrifice at 1.5, 3, 6, 9, 12 and 24 h.

2.3. Biological samples

Blood samples were collected by intracardiac punction with heparinized syringe under light gaseous anaesthesia. After centrifugation (10 min, 1547 × *g*, 10 °C), plasmas were separated in two aliquots and frozen at −70 °C before analysis.

Following sacrifice, the brains were quickly removed and dissected on an ice-cold glass plate and the parietal cortex was removed from each hemisphere. One side only was used to measure norepinephrine and its metabolites. Parietal cortices were weighed and immediately frozen at −70 °C until analysis.

2.4. Solutions of standards and internal standard

DHBA was chosen as internal standard because is not present in biological fluids and tissues and gave the same reaction at the amperometric detector as MHPG, DHPG and NE.

MHPG, DHPG, NE and DHBA solutions were prepared in mobile phase to contain 0.1 mmol/l and were stored 2 months at −24 °C. A study of standard solutions and IS stability has been done during 2 months. These solutions were further diluted to give the required concentrations.

2.5. Preparation of biological samples

One milliliter of plasma sample was first washed by hexane to remove lipids. Proteins were precipitated with sulfosalicylic acid (10 g/100 ml) and 0.1 ml of internal standard (DHBA) was added. After centrifugation, the supernatant was washed with ethylacetate saturated by sodium chloride. The ethylacetate phase containing catecholamines was evaporated to dryness at 37 °C under a stream of dry nitrogen and frozen at −24 °C until analysis. For catecholamines analysis, the residue was reconstituted in 0.1 ml of mobile phase and 20 μl were injected in the HPLC system.

Brain samples were disrupted by sonication at 4 °C during 20 min with 0.4 ml of mobile phase and 0.1 ml of internal standard. After centrifugation (20 min, 1547 × *g*, 4 °C) 20 μl of supernatant were injected immediately.

2.6. Preparation of calibration curves

For catecholamines determination in plasma and brain, different standard solutions were prepared to contain 7.0, 12.0, 19.0, 23.0 ng/ml, 6.0, 10.0, 15.0, 20.0 ng/ml, 20.0, 30.0, 50.0, 60.0 ng/ml for MHPG, DHPG and NE, respectively. Calibration curves were obtained by plotting peak-area ratios to internal standard versus the amounts of NE, MHPG or DHPG.

2.7. HPLC conditions

The HPLC system consisted of a BECKMAN 340 pump, a BECKMAN 114 M injector with a 20 μl sample loop. Separation was achieved on an ultrasphere ODS 5.0 μm C_{18}

(25 cm × 4.6 mm i.d.) column at 20 °C (BECKMAN-France).

The mobile phase consisted of 0.1 M sodium acetate, 0.02 M citric acid, 0.4 mM OSS, 0.2 mM EDTA Na_2. The pH of the buffer running solution was adjusted to 4.85 then filtered through a 0.45 μm filter (Millipore, Bedford, MA, USA). Methanol was added to give a final composition of 4.5% methanol (v/v). A flow rate of 0.8 ml/min was used in all experiments.

The electrochemical detector model ELDEC 103 (CHROMATOFIELD, France) consist of a glassy carbon working electrode and a silver/silver chloride reference electrode. A potential of +0.78 V was applied across the electrodes of the detector.

2.8. Validation criteria

2.8.1. Linearity

Working standards were prepared as described above. Four reference samples were used for calibration curves of MHPG (7.0–23.0 ng/ml), DHPG (6.0–20.0 ng/ml) and NE (20.0–60.0 ng/ml). Each determination was done six times. The calibration factors were calculated according to least-squares linear regression.

2.8.2. Precision and accuracy

Precision was determined for both inter- and intra-day variability. These measurements were made by HPLC analysis of MHPG (7.0, 12.0, 19.0 ng/ml), DHPG (6.0, 10.0, 15.0 ng/ml) and NE (20.0, 30.0, 50.0 ng/ml), on 10 consecutive days (inter-day variation or reproducibility). Ten quality control solutions at three levels were analysed during the same day for MHPG (12.5, 22.0, 28.0 ng/ml), DHPG (7.0, 12.0, 16.0 ng/ml) and NE (26.0, 45.0, 57.0 ng/ml) for intra-day variation determination (repeatability).

2.8.3. Limit of detection (LOD) and limit of quantification (LOQ)

The signal given at the retentional time (MHPG: 12.3 min, DHPG: 5.4 min, NE: 13.7 min) with a signal-to-noise ratio of 3 was determined as LOD (Fig. 1). The signal given with a signal-to-noise at 10 was determined as LOQ [33].

3. Results and discussion

Chromatograms and formulas of MHPG, DHPG and NE are shown in Fig. 1. DHPG and MHPG present a good resolution (Rs = 14.55), as well as MHPG and NE (Rs = 4.09), NE and DHBA (Rs = 24.87) (Fig. 1).

Retention factors were 1.0, 4.0, 5.1 and 16.5 for DHPG, MHPG, NE and DHBA, respectively.

The stability study of standard solutions and IS was presented in Fig. 2 plotting peak height during 2 months. No derive was observed during this time.

3.1. Linearity

To determine linearity (Fig. 3) the data were fitted to a line by the equation $y = ax + b$ where y is the area ratio, b the intercept and a the slope.

For MHPG over the range 7–23 ng/ml, for DHPG over the range 6–20 ng/ml and for NE over the range 20–60 ng/ml a linear fit was used satisfactorily with a mean r of 0.999.

3.2. Precision and accuracy

The intra-day precision (repeatability) established in the same tray on 10 assays for MHPG (12.5, 22.0, 28.0 ng/ml; CV(%) = 7.63; 3.37; 2.93), DHPG (7.0, 12.0, 16.0 ng/ml; CV(%) = 4.92; 3.74; 1.72) and NE (26.0, 45.0, 57.0 ng/ml; CV(%) = 4.25; 2.03; 1.13) gave a good precision (Table 1).

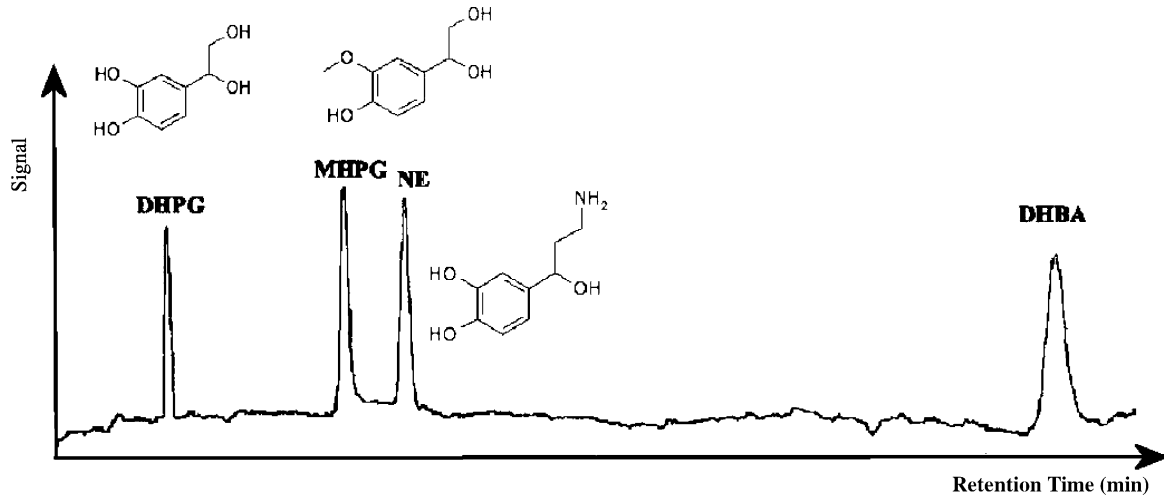

Fig. 1. Total chromatogram of MHPC, DHPC and NE.

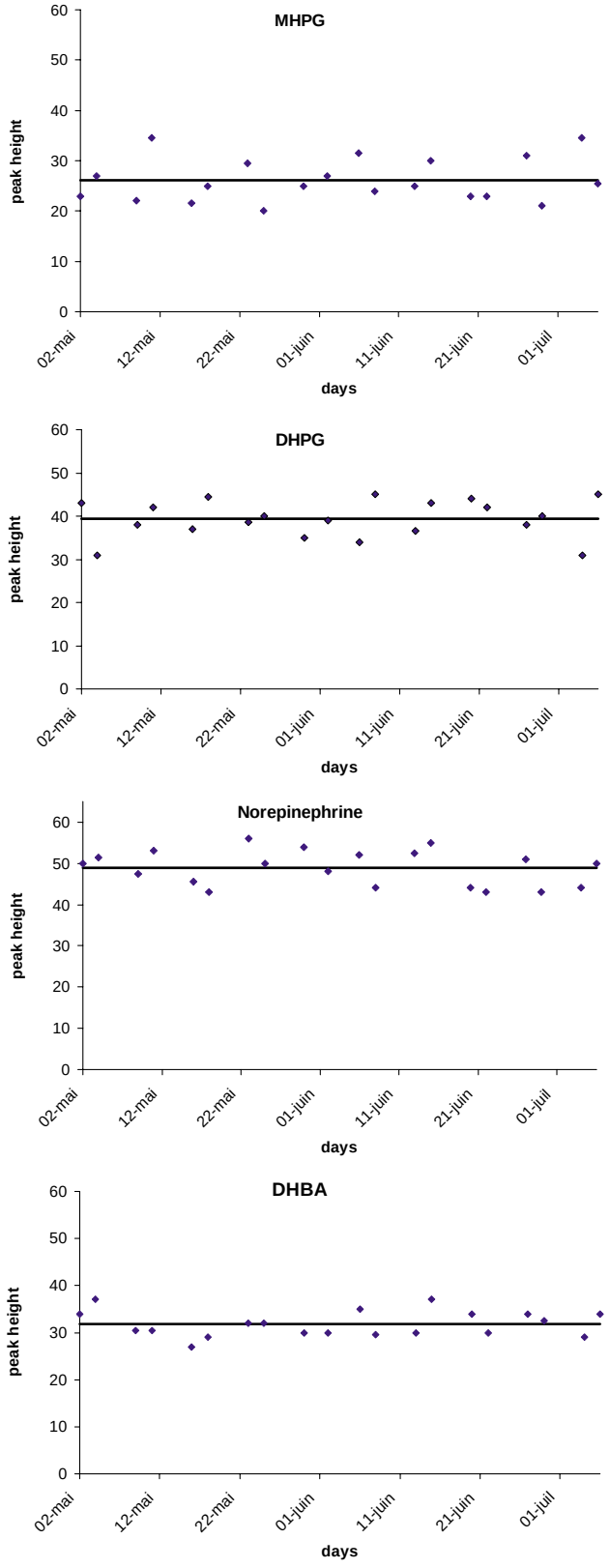

Fig. 2. Stability study of standard solutions and internal standard.

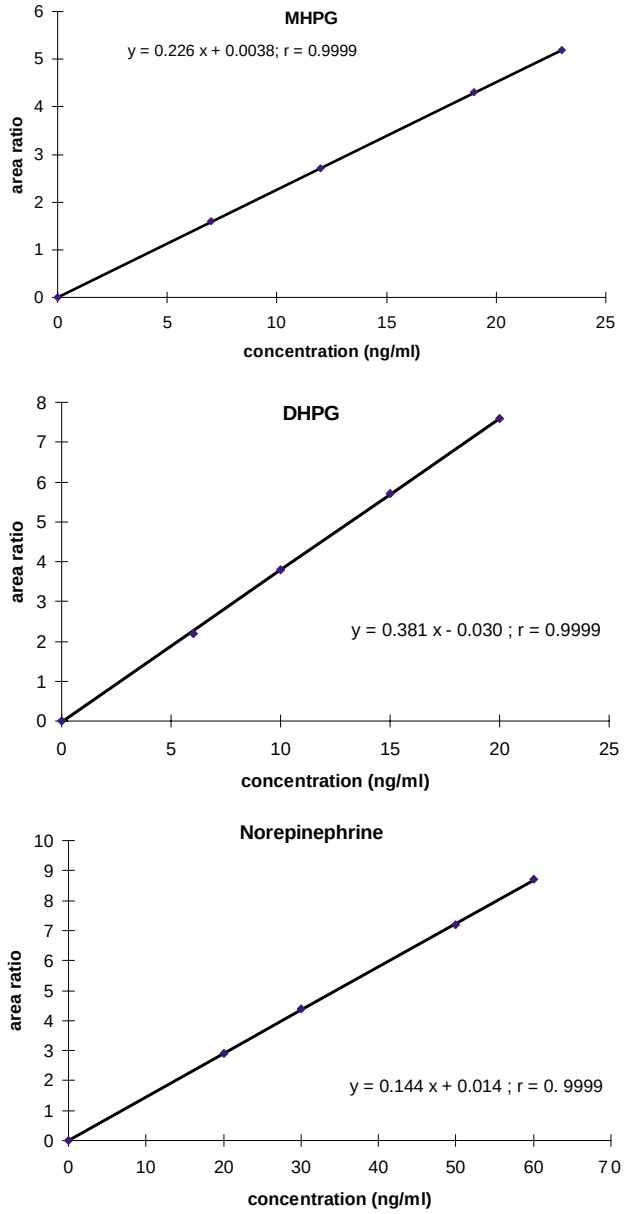

Fig. 3. Linearity of standard curves.

The inter-day precision (reproducibility) was clearly satisfactory with CVs between 5.94 and 11.0% for MHPG, between 1.97 and 4.40% for DHPG and between 2.88 and 4.35% for NE (Table 2).

These suitable CVs were obtained for MHPG in the range 7.0–19.0 ng/ml, for DHPG in the range 6.0–15.0 ng/ml and for NE in the range 20.0–50.0 ng/ml.

Figures for accuracy obtained for MHPG (mean: 98.5%), DHPG (mean: 97.2%) and NE (mean: 99.2%) from 10 days calibration curves are satisfactory.

3.3. LOD and LOQ

The LOD for MHPG, DHPG and NE analysis were, respectively, 0.5, 0.2 and 0.6 ng/ml but the precision of

Table 1
Repeatability of norepinephrine, MHPG and DHPG quantification: intra-day precision

Compounds	Theoretical concentrations (ng/ml)	*n*	Concentrations found (mean ± S.D.)	CV (%)	Accuracy (%)
Norepinephrine	26.0	10	25.1 ± 1.068	4.25	96.5
	45	10	44.5 ± 0.904	2.03	98.7
	57	10	57.3 ± 0.650	1.13	100
MHPG	12.5	10	12.3 ± 0.935	7.63	98
	22	10	21.6 ± 0.727	3.37	98.1
	28	10	27.8 ± 0.815	2.93	99.3
DHPG	7	10	6.7 ± 0.329	4.92	95.4
	12	10	11.8 ± 0.442	3.74	98.3
	16	10	15.7 ± 0.271	1.72	98.3

Table 2
Reproducibility of norepinephrine, MHPG and DHPG calibration curves during 10 consecutive days: inter-day precision

Compounds	Theoretical concentrations (ng/ml)	*n*	Concentrations found (mean ± S.D.)	CV (%)	Accuracy (%)
Norepinephrine	20	10	19.8 ± 0.861	4.35	98.8
	30	10	29.9 ± 1.090	3.64	99.7
	50	10	49.6 ± 1.430	2.88	99.3
MHPG	7	10	6.8 ± 0.751	11.0	97.4
	12	10	11.8 ± 0.974	8.28	98.0
	19	10	19.0 ± 1.130	5.94	100
DHPG	6	10	5.8 ± 0.254	4.40	96.1
	10	10	9.5 ± 0.337	3.56	94.5
	15	10	15.1 ± 0.299	1.97	101

concentration determination is poor at this very low concentration due to background noise interference. The lowest concentrations (LOQ) this method could reproducibly quantitate were 1.7, 0.7 and 2.0 ng/ml for MHPG, DHPG and NE, respectively.

3.4. Statistics

Results are presented as the mean ± S.D. of 6–10 values for each experimental hour.

Statistical significance of the mean difference between two successive experimental hours was assessed using Student's *t*-test and two ways ANOVA (factors of treatment and time).

4. Application

The method described herein was applied to quantify simultaneously NE and its two major metabolites: MHPG and DHPG in plasma and cerebral cortex of rats. Two groups of animals receiving either sodium chloride or lithium chloride (2 mmol/kg/IP) were considered. They were killed at different time over a 24 h period following salt administration.

After LiCl treatment, a decrease of NE in plasma was observed at 1.5 h with a significant increase ($P < 0.001$) at 6 h, followed by a fall up to 24 h (Fig. 4a).

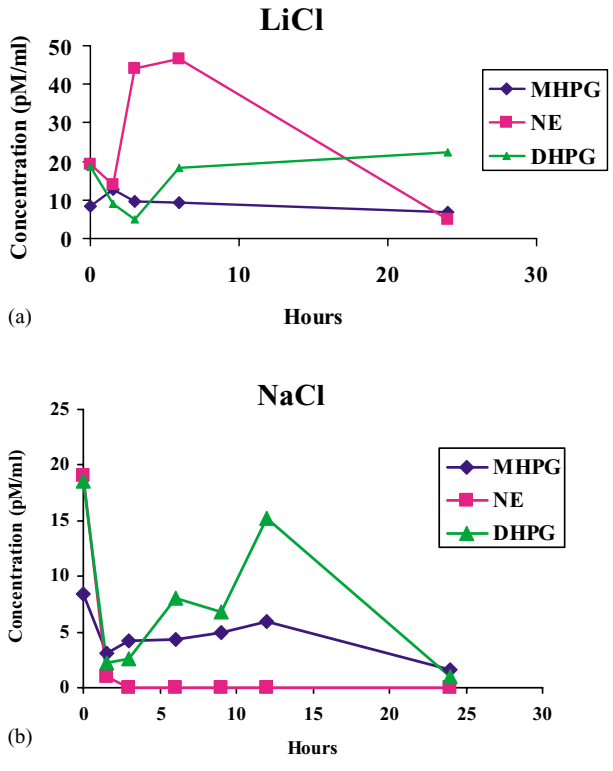

Fig. 4. Time courses of change in NE, MHPG and DHPG concentrations in rats plasma: (a) LiCl treated rats ($n = 6$) and (b) NaCl treated rats ($n = 10$).

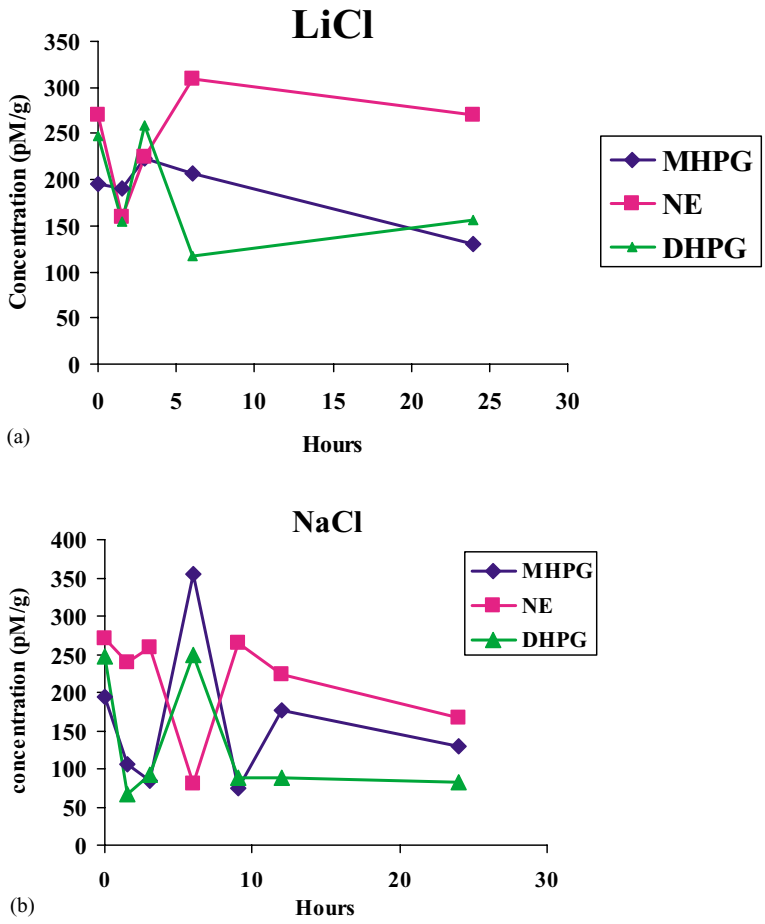

Fig. 5. Time courses of change in NE, MHPG and DHPG concentrations in rats cerebral cortex: (a) LiCl treated rats ($n = 6$) and (b) NaCl treated rats ($n = 10$).

DHPG variations were similar to NE but occurred several hours later.

An increase of MHPG occurred at 1.5 h while conversely the NE level decreased; it was then followed by a progressive decrease to the basal value reached at 24 h.

After NaCl treatment, there was a sudden fall of NE at 1.5 h in plasma (Fig. 4b). NE disappeared at 3 h. MHPG decreased more lightly at 1.5 h and then gently increased until 12 h.

DHPG as NE decreased at 1.5 h and then reached a peak at 12 h.

Animals treated by LiCl showed for NE a decrease at 1.5 h and a significant increase to reach a peak at 6 h in cerebral cortex (Fig. 5a). Then NE decreased progressively to the basal value at 24 h.

DHPG decreased at 1.5 h as NE then both of these metabolites increased to reach a peak at 3 h. Afterwards they decreased progressively to 24 h.

For animals treated by NaCl we could observe in cerebral cortex an inverted variation between NE and its metabolites with a NE significant decrease at 6 h ($P < 0.001$) and a significant increase ($P < 0.001$) for DHPG and MHPG at the same time (Fig. 5b).

At 9 h NE, MHPG and DHPG went back to level reached at 3 h. Between 9 and 12 h, while MHPG increased ($P < 0.01$) DHPG unchanged.

5. Conclusions

An HPLC-ECD method for the simultaneous determination of NE, MHPG and DHPG was developed. Advantages of this method are its practicability (minimal sample pre-treatment as well as cortex or plasma), best specificity/selectivity and good accuracy and precision of results. It should prove very useful for rapid determination of plasma NE, MHPG and DHPG levels in manic depressive patients treated by lithium and then allow to try to evaluate the relationship between lithium turnover and its physiological effects.

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