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Simultaneous liquid chromatographic analysis of catecholamines and 4-hydroxy-3-methoxyphenylethylene glycol in human plasma Comparison of amperometric and coulometric detection

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Abstract

The comparison of two HPLC methods, one with electrochemical detection and the other with coulometric detection, for the simultaneous analysis of catecholamines and 4-hydroxy-3-methoxyphenylethylene glycol (MHPG) in human plasma is presented. The careful pre-treatment of plasma samples is based on an innovative two-step procedure by means of solid-phase extraction (SPE) which uses one single hydrophilic–lipophilic balance cartridge. The extraction yield values found were higher than 85% for epinephrine, norepinephrine and MHPG, and higher than 70% for dopamine. The assays carried out on real plasma samples with the coulometric system gave good results in terms of sensitivity (limits of quantitation: 0.10–0.15 ng ml^{-1} for catecholamines, 0.6 ng ml^{-1} for MHPG) and selectivity, while interference was sometimes found when using the amperometric system. Precision was also satisfactory, with relative standard deviation values for intermediate precision always lower than 6%. The HPLC method with coulometric detection coupled to a novel SPE procedure is thus suitable for the simultaneous determination of catecholamines and MHPG in plasma of volunteers subjected to experimental stress.

Keywords: Detection, LC; Catecholamines; Hydroxymethoxyphenylethylene glycol

1. Introduction

Epinephrine (E, Fig. 1a), norepinephrine (NE, Fig. 1b) and dopamine (DA, Fig. 1c) are the main endogenous catecholamines (CAs) which can act as hormones and/or neurotransmitters in several physiological and pathological situations related to both the autonomic and central nervous systems. Thus, the determination of CA levels in biological fluids is a useful tool for the correct diagnosis of related diseases. The main metabolite of NE is 4-hydroxy-3-methoxyphenylethylene glycol (MHPG, Fig. 1d). High plasma levels of this compound [1,2] are associated with states of anxiety in humans, are a marker for neuroblastoma [3] and are used for the diagnosis of cardiovascular diseases [4]. From all this stems the need to have reliable analytical methods available for the determination of CAs and MHPG in human plasma.

Since CAs are electroactive compounds, several papers [5–16] have been published on their analysis in biological fluids by means of HPLC methods with electrochemical detectors. Fluorimetric detection after derivatisation [17] or chemiluminescence detection [18,19] have also been reported. Only a few papers have been published on the analysis of MHPG in plasma, based on HPLC with fluorimetric [20], amperometric [21,22] or coulometric [23] detection. Since MHPG is chemically rather different from CAs, their determination is usually carried out separately on different chromatographic systems. To our knowledge, only one recent paper [24] simultaneously determines CAs and some metabolites by HPLC with coulometric detection. This method uses one plasma sample, however, the analytes are extracted by means of two solid-phase extraction (SPE) procedures using two different cartridges, namely one hydrophilic–lipophilic balance (HLB) cartridge and one C_{18} cartridge. In this paper, we compare the performance

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of two different HPLC methods, one with amperometric detection and other with coulometric detection, for the simultaneous analysis of E, NE, DA and MHPG. Both methods are coupled to an original, sequential double SPE procedure using only one HLB cartridge. This study is a part of a broader interdisciplinary research on neuroendocrine responses to experimentally induced psychological stress or aggressiveness [25–29] in healthy humans and in subjects with substance abuse problems.

2. Experimental

2.1. Chemicals

Dopamine, norepinephrine, epinephrine hydrochloride, 4-hydroxy-3-methoxyphenylethylene glycol, dihydroxybenzylamine hydrobromide (internal standard (IS), Fig. 1e) for the quantitation of CAs, diphenylboric acid 2-aminoethyl ester (DPB), tetrabutylammonium bromide (TBA) and bovine albumin were purchased from Sigma (St. Louis, MO, USA). Ethylenediaminetetraacetic acid sodium salt (EDTA), citric acid, 1 mol l^{-1} sodium hydroxide, potassium chloride (KCl), sodium chloride (NaCl), ammonium chloride (NH_4Cl), sodium metabisulphite, 30% (w/w) concentrated ammonia, 85% (w/w) phosphoric acid and 37% (w/w) hydrochloric acid, all analytical grade, were purchased from Carlo Erba (Milan, Italy); methanol for HPLC and 1-octanesulphonic acid sodium salt monohydrate (OSA) were purchased from Fluka (Buchs, Switzerland). Ultrapure water (18.2 MΩ cm) was obtained from a Millipore (Milford, MA, USA) Milli-Q apparatus.

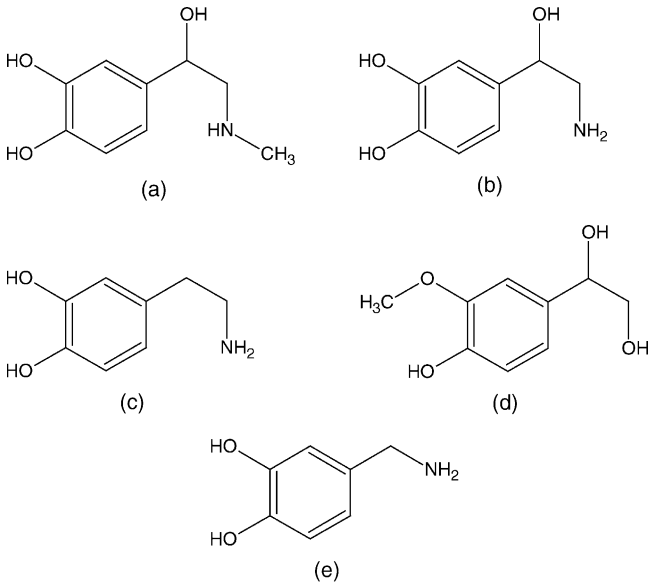

Fig. 1. Structural formulae of: (a) epinephrine; (b) norepinephrine; (c) dopamine; (d) 4-hydroxy-3-methoxyphenylethylene glycol; (e) dihydroxybenzylamine hydrobromide.

2.2. Solutions

Stock solutions (1 mg ml^{-1}) of E, NE, MHPG, DA and IS were prepared by dissolving 20 mg of pure substance, 160 mg of NaCl, 20 mg of sodium metabisulphite and 100 μl of 37% HCl in 20 ml of ultrapure water. This saline solution prepared at acidic pH and containing an antioxidant is useful to avoid analyte degradation, as previously reported [15]. Standard solutions were obtained by diluting stock solutions with ultrapure water.

Buffer solution A was prepared by dissolving 600 mg of TBA, 250 mg of EDTA and 5.35 g of NH_4Cl, in this precise order, in about 30 ml of water; the solution was then buffered up to pH 8.5 with 30% ammonia and 100 mg of DPB powder were added. The mixture was finally stirred for 15 min maintaining the pH at 8.5. The solution was then brought up to 50 ml with ultrapure water and stored at 4 °C for one night; in the morning, the solution was buffered again at pH 8.5 with 30% ammonia. This solution is stable for about 3 months at 4 °C.

Buffer solution B was prepared as follows: 200 mg of TBA, 25 mg of EDTA and 535 mg of NH_4Cl were dissolved in about 30 ml of water and brought up to pH 8.5 with 30% ammonia, then the solution was brought up to 50 ml with ultrapure water.

The "*reconstituted*" *plasma* was prepared by dissolving 800 mg of NaCl, 20 mg of KH_2PO_4, 115 mg of Na_2PO_4, 20 mg of KCl and 4 g of bovine albumin in 100 ml of ultrapure water.

2.3. HPLC methods

2.3.1. Chromatographic conditions

The chromatographic separation was achieved on a Varian (Harbor City, CA, USA) Microsorb reversed-phase column (C_8, 250 mm × 4.6 mm i.d., 5 μm), with a Phenomenex Security Guard precolumn (C_8, 4.0 mm × 3.0 mm i.d.). The mobile phase was a mixture of 5% of methanol and 95% of an aqueous solution of 10.5 g l^{-1} citric acid, 20 mg l^{-1} EDTA and 20 mg l^{-1} OSA buffered at pH 3.5 with 1 mol l^{-1} NaOH. The flow rate was 1 ml min^{-1}.

The samples were injected by means of a 20 μl loop for the coulometric detector and a 50 μl loop for the amperometric detection.

2.3.2. Detection systems

Coulometric system: a Beckman Instruments (Palo Alto, CA, USA) 168 chromatographic pump with an ESA (Milford, MA, USA) Coulochem II coulometric detector (the conditioning cell was set at 0 mV, while the analytical cell was set at detector 1 = +450 mV and detector 2 = −350 mV).

Amperometric system: a Varian model 9002 chromatographic pump with an Antec (Leiden, The Netherlands) Decade amperometric detector (set at +800 mV, 30 °C; working electrode: glassy carbon; reference electrode: Ag/AgCl; auxiliary electrode: stainless steel 316).

Data obtained with the Coulochem II detector were analysed by means of Beckman "Gold Noveau" software installed on a Intel Pentium processor while data obtained with the Decade amperometric detector were handled by means of Varian "Star Chromatography" software installed on a Intel Pentium processor.

2.4. Extraction procedure

The cartridges used for the SPE step were Waters (Milford, MA, USA) Oasis HLB (30 mg, 1 ml). The extraction of CAs and MHPG from plasma was carried out following three steps.

2.4.1. Step I: MHPG extraction

Different amounts of CAs and MHPG (as standard solutions), 5 ng ml^{-1} of IS, 100 μl of 1 M hydrochloric acid and 300 μl of ultrapure water were added to 500 μl of plasma (human or reconstituted), and loaded onto an Oasis HLB cartridge previously activated with 1 ml of methanol two times and conditioned with 1 ml of water two times. The cartridge was washed with 300 μl of water; loading and washing solutions were collected in a 5 ml glass tube (solution 1). Then, the cartridge was washed with 1 ml of water two times and the elution was carried out with 300 μl of MeOH. This eluate containing MHPG was brought to dryness in a rotary evaporator at 37 °C.

2.4.2. Step II: catecholamine extraction

The Oasis cartridge was washed two times with 1 ml of methanol and two times with 1 ml of water, then conditioned with 1 ml of methanol two times, 1 ml of water two times and 1 ml of buffer solution B two times. An aliquot of 300 μl of buffer B was added to solution 1 and mixed, then 1.2 ml of buffer solution A (containing 8.8 mM of the DPB complexing agent for CAs) was added. The resulting mixture was loaded onto the previously used and conditioned cartridge. After loading, the cartridge was washed with 1 ml of buffer A, then 1 ml of a buffer B–methanol (1:1) mixture twice. The elution was carried out with 500 μl of the mobile phase.

2.4.3. Step III: merging of the two extract solutions

The dried residue of the MHPG eluate was redissolved in the eluate obtained in step II that contains the CAs. Then, 20 or 50 μl of the resulting solution were injected into the HPLC system (coupled to an amperometric or coulometric detector) for simultaneous analysis of CAs and MHPG.

2.5. Extraction yield

Known amounts of CAs and MHPG and constant amounts of IS standard solutions were added to 500 μl of reconstituted plasma to obtain concentrations of 0.5, 1.0 and 2.0 ng ml^{-1} of the analytes, then the mixture was subjected to the extraction procedure described earlier, to obtain extraction yield values.

2.6. Sample collection

Blood samples were drawn into test tubes containing EDTA; 100 μg ml^{-1} sodium metabisulphite were added and the blood was centrifuged at 3000 rpm for 20 min. The supernatant plasma was transferred into test tubes and frozen at −80 °C until analysis.

2.7. Method validation

2.7.1. Calibration curves

Standard solutions of the four analytes and the IS were added to 500 μl of reconstituted plasma to obtain concentrations in the 0.2–10.0 ng ml^{-1} range for CAs and in the 1.0–30.0 ng ml^{-1} range for MHPG, then the mixtures were subjected to the extraction procedure described earlier and injected into the HPLC system. The six-point calibration curves were calculated plotting the analyte/IS peak area ratio values against the analyte concentrations. Limits of quantitation (LOQ) and detection (LOD) were evaluated for both HPLC systems according to the USP XXIV guidelines [30].

2.7.2. Precision

The SPE procedure described earlier was carried out on plasma samples spiked with the analytes: six times during the same day to obtain relative standard deviation (R.S.D., %) values for repeatability (intraday precision), and six times over six different days to obtain R.S.D. values for intermediate precision (interday precision). These values were obtained at three different concentration levels, namely: 0.5, 1.0 or 2.5 ng ml^{-1} for each catecholamine and 4.0, 8.0 or 12.0 ng ml^{-1} for MHPG.

2.7.3. Accuracy

Known amounts of CAs, MHPG and IS standard solutions were added to known amounts of real plasma samples, which had already been analysed. The method accuracy was evaluated by calculating the difference between the spiked sample peak area ratios and the original sample peak area ratios, then comparing these differences with the peak areas obtained by injecting standard solutions having the same concentration as the sample spiking.

3. Results and discussion

3.1. Chromatographic conditions

Some chromatographic parameters such as column type, mobile phase and conditioning time were investigated to obtain a good separation of the four analytes within an acceptable time span. Differently from other papers [21,24], which use C_{18} stationary phases, we chose to use a C_8 column to obtain good separation in a relatively short times. With this column, however, the mobile phase already used for the

analysis of CAs [15] did not allow for the separation of NE and MHPG and that used for the analysis of MHPG [23] did not retain NE. Thus, the percentage of methanol (between 2.5 and 8.0%) and the pH value (in the 2.5–4.0 range) of the mobile phase were studied. Best chromatographic performance was obtained with a mobile phase at pH 3.5 containing 5.0% of methanol. At least 4 h are necessary to reach an equilibrium between the mobile phase containing OSA and the stationary phase; if samples are injected earlier, retention times vary considerably, thus impairing correct peak identification.

Regarding the electrochemical detection, the potential values already established in our previous papers [15,16,22,23] were used (see Section 2).

3.2. SPE procedure development

Several sample pre-treatments are reported in the literature for the analysis of CAs based on different retention mechanisms: for example, anion exchange [16], reversed-phase extraction after complexing of CA with DPB [12,15,24], direct-phase extraction by means of alumina cartridges [7,10]. To our best knowledge, only three papers use an SPE procedure for MHPG extraction from the matrix, and all of them utilise Oasis HLB cartridges [22–24]. Starting from the procedures we previously implemented to separately extract CAs [15] and MHPG [23], we tried to develop a sequential SPE procedure for the extraction of all analytes from the same plasma sample using the same Oasis HLB cartridge. It is able to retain MHPG due to its hydrophilic–lipophilic balance, while it does not retain CAs, which are then reloaded onto the same cartridge after suitable complexation. The entire extraction procedure consists of three steps.

3.2.1. Step I

The extraction procedure of MHPG from plasma was carried out as previously described [23], with some minor modifications (see Section 2). In these conditions MHPG was adsorbed onto the resin, while CAs and the IS flowed out of the cartridge during the loading and washing steps (solution 1). MHPG was then eluted from the cartridge with MeOH and the eluate evaporated to dryness.

3.2.2. Step II

Since the complexation reaction of CAs with DPB requires a basic pH, buffer B is added to solution 1 (which is acidic), followed by the addition of buffer A (containing the DPB complexing agent). This solution is then loaded onto the already used Oasis cartridge (which in the meantime has been suitably washed and conditioned). The conditioning step is different from that used in our previous paper [15]; in fact, buffer A was substituted with buffer B to avoid overloading the sorbent with excess DPB (which is present in a large amount in the loading mixture). The elution was carried out by passing 500 μl of mobile phase through the cartridge; the mobile phase is acidic and thus disrupts the complex and allows to obtain free CAs.

3.2.3. Step III

The dry extract of MHPG (from step I) was redissolved in the eluate (step II) to obtain a solution containing all the analytes, which is then injected into the two HPLC systems.

3.3. Method validation

Since CAs and MHPG are endogenous substances, method validation was carried out using samples of reconstituted plasma (see Section 2) as “blank human plasma”. These samples spiked with different concentrations of CAs and MHPG were subjected to the procedure described earlier. The analyte/IS peak area ratios were compared to the area ratios of standard solutions at the same nominal concentration, obtaining extraction yield values. The chromatograms obtained from a reconstituted plasma sample spiked with 2 ng ml^{-1} of CAs and IS and 10 ng ml^{-1} of MHPG, injected into the coulometric and the amperometric systems, are shown in Fig. 2a and b, respectively. NE, MHPG, E, IS and DA were detected at retention times of 8.1, 10.1, 11.1, 13.7 and 22.0 min, respectively. No interference was found at retention times corresponding to those of the analytes. Table 1 shows the values of the extraction yield obtained for three different concentrations of the analytes and the corresponding relative standard deviation values. As can be seen, the values of absolute recovery are satisfactory; for E, NE and MHPG, in particular, the extraction yield values are higher than 85%, and for DA they are higher than 75%. These values are similar to those reported in the literature (about 65% for DA, 75% for NE and 85% for E and MHPG [24]). The precision data are also satisfactory considering the very low concentrations investigated: the R.S.D. values are always lower than 3.5%

Table 1
Extraction yield results for CAs and MHPG, as obtained by means of the coulometric system (precision data are also reported)

Analyte	Amount added (ng ml^{-1})	Mean extraction yield (%)[a]	Intermediate precision (R.S.D., %)[a]
NE	0.5	89 (88)[b]	5.6 (5.8)[b]
	1.0	91 (92)	3.9 (3.7)
	2.5	96 (95)	1.2 (1.2)
MHPG	4.0	102 (101)	4.1 (4.3)
	8.0	95 (96)	3.7 (3.8)
	12.0	99 (97)	2.9 (2.8)
E	0.5	87 (84)	4.8 (5.1)
	1.0	86 (85)	3.6 (3.9)
	2.5	85 (87)	2.9 (3.0)
DA	0.5	77 (77)	5.9 (6.0)
	1.0	78 (76)	5.5 (5.7)
	2.5	72 (73)	3.2 (3.3)

[a] $n = 6$.
[b] The data in parentheses are relative to the amperometric system.

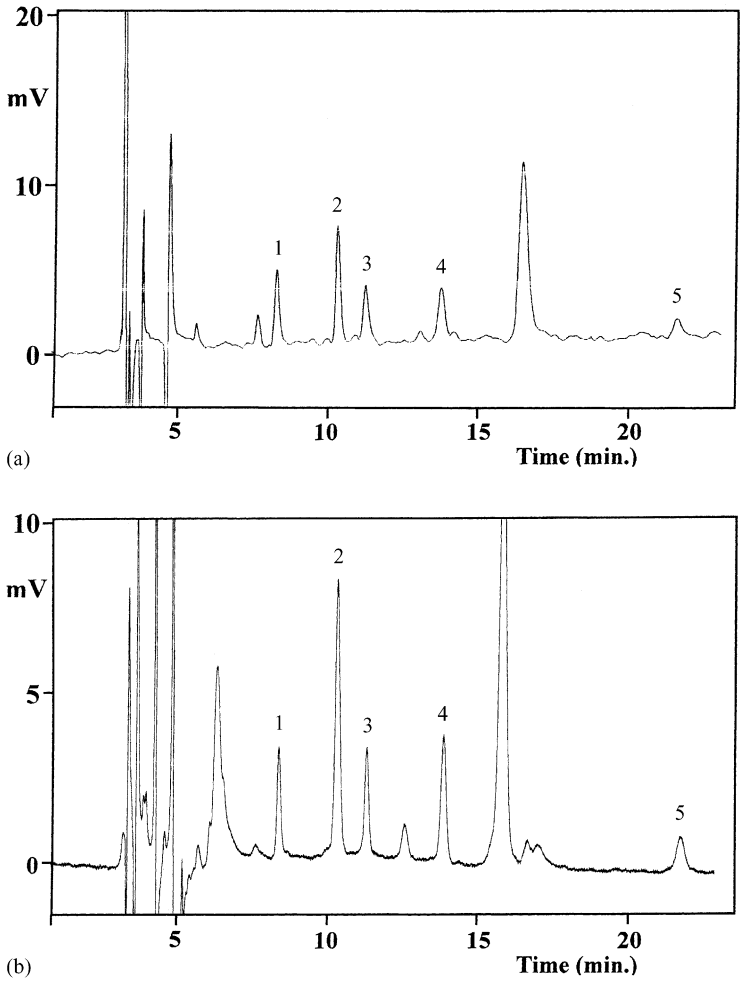

Fig. 2. Chromatograms of a blank plasma sample spiked with 2 ng ml^{-1} of NE, E and DA, 10 ng ml^{-1} of MHPG and 2 ng ml^{-1} of the IS, obtained by means of: (a) the coulometric system; (b) the amperometric system. Peak identification: (1) NE; (2) MHPG; (3) E; (4) IS; (5) DA.

for the high concentrations and lower than 6.5% for the low concentrations.

Linearity was found in the 0.2–10.0 ng ml^{-1} range for CAs and in the 1.0–30.0 ng ml^{-1} range for MHPG. Regression parameters are reported in Table 2 together with the LOQ and the LOD. It can be observed that these limits are suitable in all cases for the purpose of analysing CAs and MHPG in human plasma. The use of a larger loop (50 μl) for amperometric detection than that (20 μl) used for coulometric detection allowed to obtain limits of detection and quantitation that are roughly comparable for the two methods, even though it is known that the coulometric detector has much better intrinsic sensitivity.

3.4. Application to human plasma samples

The method was applied to plasma samples from young volunteers subjected to emotional stress. The chromatogram (Fig. 3a) was obtained from a plasma sample analysed by means of the coulometric system; as one can see, all four analytes give neat and resolved chromatographic peaks. The analyte concentrations found were: 1.17, 10.53, 0.78 and

Table 2
Linearity parameters

Analyte	Amperometric detector				Coulometric detector			
	Regression equation	r^2	LOD (ng ml^{-1})	LOQ (ng ml^{-1})	Regression equation	r^2	LOD (ng ml^{-1})	LOQ (ng ml^{-1})
NE	$y = 374 + 7251x$	0.9950	0.10	0.20	$y = 532 + 69133x$	0.9980	0.05	0.10
E	$y = 3146 + 7598x$	0.9952	0.10	0.20	$y = 1312 + 68179x$	0.9968	0.05	0.10
DA	$y = -4794 + 5669x$	0.9944	0.15	0.20	$y = -1022 + 59318x$	0.9936	0.06	0.12
MHPG	$y = -668 + 8003x$	0.9978	0.30	0.60	$y = -419 + 21809x$	0.9978	0.20	0.50

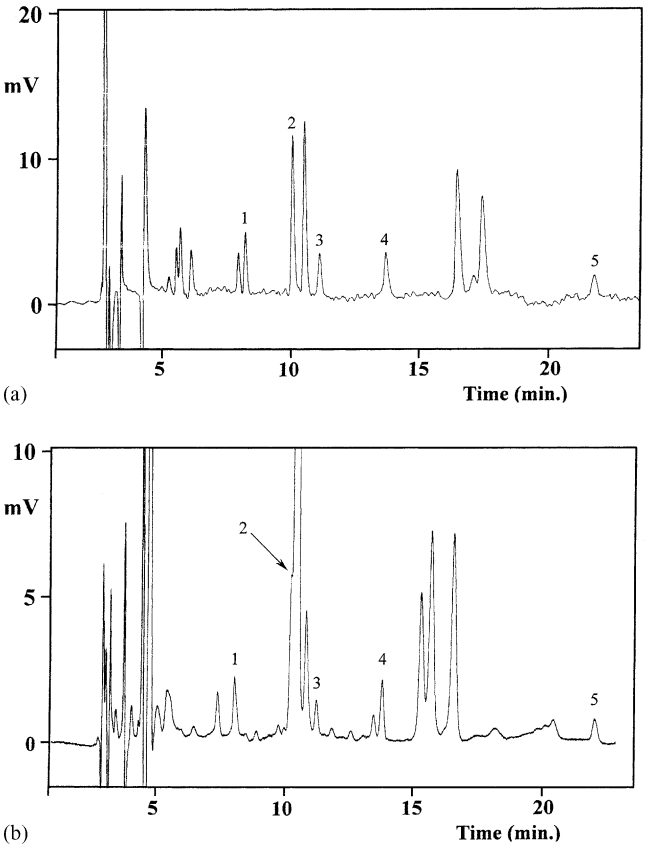

Fig. 3. Chromatograms of a human plasma sample spiked with 2 ng ml^{-1} of the IS, obtained by means of: (a) the coulometric system; (b) the amperometric system. Peak identification as in Fig. 2.

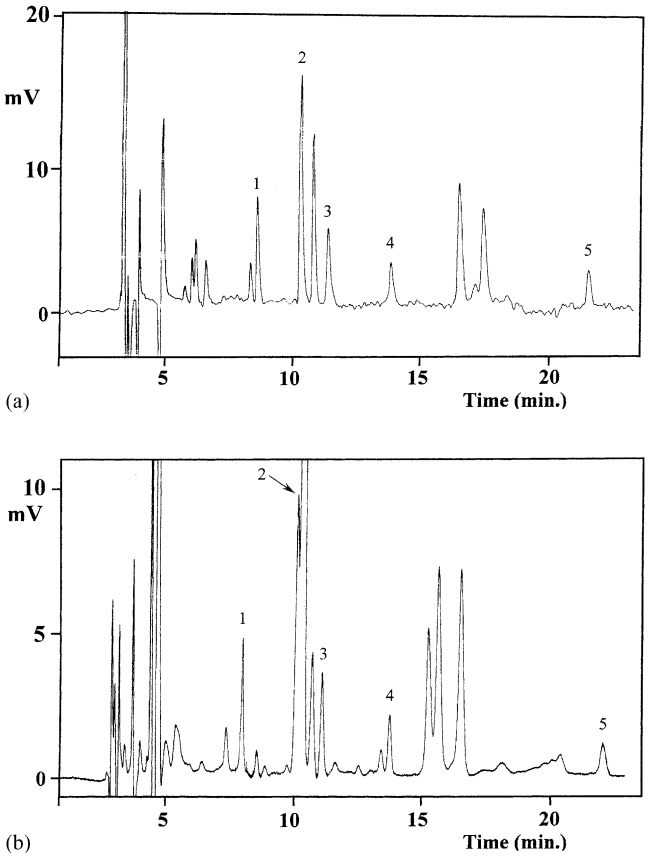

Fig. 4. Chromatograms of the same sample of human plasma sample as in Fig. 3, spiked with 1.0 ng ml^{-1} of E, 0.5 ng ml^{-1} of NE, 10.0 ng ml^{-1} of MHPG, and 0.5 ng ml^{-1} of DA, obtained by means of: (a) the coulometric system; (b) the amperometric system. Peak identification as in Fig. 2.

0.56 ng ml^{-1} for NE, MHPG, E and DA, respectively. Similar results were obtained by means of the amperometric system (Fig. 3b), although the presence of an interference peak which partially overlaps that of MHPG, is apparent in the latter chromatogram.

The accuracy of the method was verified by means of recovery studies, adding known amounts of CA and MHPG standard solutions to a known amount of (already analysed) human plasma and subjecting the mixture to the usual extraction procedure. An example is reported in Fig. 4 (a: coulometric detector; b: amperometric detector), where the same plasma sample of Fig. 3 was spiked with 1.0 ng ml^{-1} of E, 0.5 ng ml^{-1} of NE, 10.0 ng ml^{-1} of MHPG and 0.5 ng ml^{-1} of DA. The accuracy data resulted good for all analytes: 90% for NE, 89% for E, 96% for MHPG (coulometric only) and 81% for DA ($n = 6$).

One can note in Fig. 4b that after MHPG spiking a new peak corresponding to MHPG appears near the interference peak, however, its quantitation is difficult. Thus, the amperometric detection system allows for the determination of CAs but not always of MHPG, while the coulometric detection system gives good results for all four analytes. In fact, the coulometric detector is more selective than the amperometric one: the coupling of oxidation and reduction reactions cuts off many substances that do not have a reversible oxidative process.

Other analyses were also carried out on plasma samples taken from healthy volunteers not subjected to stress, using only the coulometric system. The mean "basal" CA and MHPG levels were found to be: 0.3 ng ml^{-1} for NE, 0.2 ng ml^{-1} for E, 0.2 ng ml^{-1} for DA, 5 ng ml^{-1} for MHPG. The accuracy assays carried out on these samples also gave good results (mean recovery of 90% for E and NE, 80% for DA and 95% for MHPG).

4. Conclusion

Two HPLC methods (one amperometric and one coulometric) which use the same chromatographic system for the simultaneous analysis of CAs and MHPG have been developed and compared. A novel SPE pre-treatment of plasma samples has also been implemented which requires only 500 μl of plasma, using one single cartridge and a two-step procedure for the extraction of CAs and MHPG from the biological matrix. Good results for all analytes in terms of linearity, extraction yield and precision were obtained with

both detectors. The coulometric detector demonstrated better performance for sensitivity and selectivity than the amperometric detector. The procedure herein proposed needs only one chromatographic column and the analysis of CAs and MHPG is carried out with one single injection. Compared with the only other paper which reports an HPLC method with an SPE procedure for the simultaneous determination of CAs and MHPG [24], the present method showed comparable precision and shorter chromatographic runs, and needs only one SPE cartridge, thus leading to a reduction in costs.

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