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Micellar liquid chromatography determination of some biogenic amines with electrochemical detection

D. Bose[1], A. Durgbanshi[1], M.E. Capella-Peiró, M. Gil-Agustí, J. Esteve-Romero, S. Carda-Broch*

Química Analítica, C.C.E.E., E.S.T.C.E., Universitat Jaume I, Av. Vicent Sos Baynat, 12071 Castelló, Spain

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Abstract

A simple and reliable liquid chromatographic procedure is successfully applied to the simultaneous determination of the biogenic amines, dopamine, serotonin, their metabolites (homovalinic acid (HVA) and hydroxyindoleacetic acid (HIAA)) as well as tyramine in serum samples. After an optimization procedure using a C18 column, the mobile phase selected was 0.15 M sodium dodecyl sulfate buffered at pH 3, in which the serum samples were directly injected and the analysis time for the five substances was less than 12 min. The use of electrochemical (ED) and ultraviolet (UV) detection was compared. The limits of detection of the biogenic amines studied were drastically improved using ED detection. Repeatability and intermediate precision were tested at three different concentrations and the relative standard deviations were below 1.5% for most assays. Finally, the method was successfully applied to the determination of biogenic amines in serum samples.

Keywords: Dopamine; Serotonin; HVA; HIAA; Tyramine; Serum; MLC; Electrochemical detection

1. Introduction

Biogenic amines (BAs), are a group of naturally occurring amines derived by enzymatic decarboxylation of the natural amino acids. Many have powerful physiological effects (e.g., histamine, serotonin, epinephrine, tyramine). Those derived from aromatic amino acids, and also their synthetic analogs (e.g., amphetamine), are of use in pharmacology. BAs such as dopamine and serotonin, are characterized by a phenyl ring containing an alkylamine chain or a carboxylic chain, as in homovalinic acid (HVA) and hydroxyindoleacetic acid (HIAA) (Fig. 1). Dopamine and serotonin are two of the most widely studied neurochemicals. Dopamine is used for the correction of hemodynamic disorders associated with shock episodes [1]. The effects of serotonin are felt most prominently in the cardiovascular system, with additional effects in the respiratory system and the intestines. HVA, the dopamine metabolite, has been proposed as a diagnostic index for neuroblastoma. The serotonin metabolite, HIAA, has also been observed in serum samples of patients, but is usually largely put down to dietary amines. Therefore, monitoring HVA and HIAA in serum, or in urine from patients whose dietary amines are well controlled, may provide a valuable diagnostic index for neuroblastoma. The serum level determination of these BAs and their metabolites is necessary in studies aimed at evaluating neuroendocrine disorders [2], and the role of the autonomic nervous system in several physiological and pathological situations in humans [3].

Tyramine is a natural substance formed from the breakdown of protein as food ages and is found in stale, fermented, or spoiled foodstuffs. The intake of food containing an excessive amount of tyramine, is generally symptomized by migraine, or it may be due to pheochromocytoma, a neuroendocrine tumor arising from chromaffin cells.

* Corresponding author. Tel.: +34 964 728 092; fax: +34 964 728 066.
E-mail address: scarda@exp.uji.es (S. Carda-Broch).
[1] On a research visit from the Department of Criminology and Forensic Science, Dr. Hari Singh Gour University, Sagar, M.P., India.

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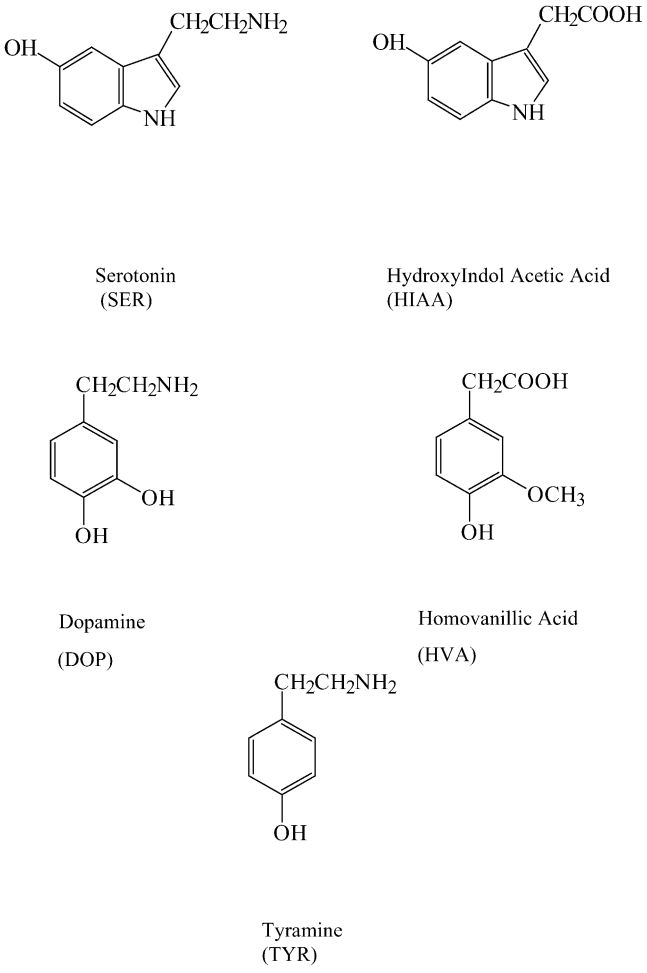

Fig. 1. Structure of the drugs studied: hydroxyindoleacetic acid (HIAA), homovalinic acid (HVA), serotonin (SER), dopamine (DOP) and tyramine (TYR).

It is therefore necessary to have a fast, reliable, and selective method for the simultaneous quantification of a mixture of these compounds to know whether the real cause of increasing amounts of BAs in blood is due to dietary amine, medicine or carcinoid tumors. High performance liquid chromatography (HPLC) has been reported for the analysis of BAs and their metabolites in biological fluids [4–9]. Several authors use an HPLC procedure with either amperometric [10,11] or coulometric [12] detection and a C18 column for the analysis of BAs in serum or urine samples. Other HPLC procedures applied to biological samples include precolumn BA oxidation to aminochromes with spectrometric or coulometric detection [13–16].

HPLC with electrochemical detection (ED) seems to be particularly suitable for the assay of these biomolecules due to its high sensitivity and selectivity. However, the complexity of matrixes makes it necessary to extract them from biological samples. Despite the large number of works published about this issue, there are still problems associated with the sample cleanup procedure. The BAs in biological samples are usually purified by adsorption on alumina [17–20] or on boric acid gel [21], or by extraction with organic solvents [22,23]. However, these methods are not selective enough and they often give poor recoveries.

Micellar liquid chromatography (MLC), which uses a surfactant solution with a concentration above the critical micellar concentration as the mobile phase, constitutes an alternative to conventional HPLC [24–26]. The simultaneous elution of hydrophobic and hydrophilic analytes is possible [27,28] without needing a gradient elution, and direct injection of physiological samples becomes feasible due to the solubility of proteins in the micelles. MLC has proved to be a useful technique in the determination of diverse groups of compounds [29–33].

The purpose of this work is to develop a rapid, simple and selective MLC procedure for the screening of dopamine, serotonin, HVA, HIAA and tyramine in serum, using a C18 column and electrochemical and UV detection. The determination of these compounds is greatly simplified by direct injection of the serum samples in the MLC system, and the use of ED reduces the protein band, thus increasing selectivity.

2. Experimental

2.1. Reagents

Sodium dodecyl sulfate (99% purity, Merck, Darmstadt, Germany), sodium dihydrogen phosphate, potassium chloride (Panreac, Barcelona, Spain), HCl, NaOH (Probus, Badalona, Spain), and methanol (Scharlab, Barcelona) were used. The biogenic amines and their metabolites (serotonin, dopamine, HVA, HIAA and tyramine) were purchased from Sigma (St. Louis, MO, USA). Stock solutions containing 100 μg/mL of the compounds were prepared in distilled-deionized water (Barnstead, Sybron, Boston, MA, USA), and suitably diluted for analysis. The micellar mobile phases and the BA solutions were filtered through 0.45 μm Nylon membranes (Micron Separations, Westboro, MA). The micellar mobile phase recommended in this work for the analyses of the BAs was 0.15 M SDS–0.01 M NaH_2PO_4–0.001 M KCl, buffered at pH 3. The pH was measured after the addition of the SDS, and no effect was observed in the performance of the pH electrodes.

2.2. Apparatus

Absorbance measurements were obtained with a Perkin Elmer UV–vis–NIR spectrophotometer (Model Lambda 19, Norwalk, CT, USA). The pH was measured with a Crison potentiometer (Model micropH 2001, Barcelona), equipped with a combined Ag/AgCl/glass electrode.

An Agilent Technologies chromatograph (Model HP 1100, Palo Alto, CA, USA), equipped with a quaternary pump, an autosampler, a UV–vis and electrochemical (Model HP 1049A) detectors, was used. Monitoring was performed

at 280 nm in the UV detector, and at 700 mV in the electrochemical detector. An Ag/AgCl electrode served as a reference in the electrochemical detector while glassy carbon was the working electrode. A Kromasil C18 column (Scharlab, 5 μm particle size, 120 mm × 4.6 mm i.d.) was employed for the analytical separation. Injection of the solutions into the chromatographic system was performed through a Rheodyne valve (Cotati, CA, USA). The flow-rate and the injection volume were 1.0 mL/min, and 20 μL, respectively. The dead time was determined as the mean value of the first significant deviation of the baseline in the chromatograms of the analytes. The signal was acquired by a PC computer connected to the chromatograph, through an HP Chemstation.

2.3. Sample preparation

The analyses were performed with 1 mL of the serum samples, which were diluted in a ratio of 1:5 with the mobile phase before injection. The serum solutions of the BAs were injected into the chromatograph without any pretreatment other than filtration, which was carried out directly into the autosampler vials through 0.45 μm Nylon membranes. The filters were previously conditioned by passing a small amount of the analyte solution through them. The optimum mobile phase was obtained using spiked serum samples containing accurately known amounts of the analytes. Several mobile phases were tested at increasing concentrations of SDS from 0.025 to 0.15 M.

3. Results and discussion

3.1. Optimization of the oxidation potential

Hydrodynamic voltammograms were plotted in order to obtain the optimum detection potential (Fig. 2). Generally, the BAs were oxidized at potentials more positive than 0.2 V. The sensitivity of the analytes increased in parallel to the applied potential up to 0.7 V, except for tyramine which does not increase in a prominent manner up to 0.7 V. The sensitivity for tyramine increased exponentially after 0.7 V, while for the rest of the analytes it remained constant (Fig. 2). However, reproducibility was bad and baseline drift was high. This is probably due to the contamination of the electrode surface by oxidation products. Thus, the detection potential was set at 0.7 V, since this value also enabled us to reduce the band of non-retained substances eluting with the dead volume.

3.2. Selection of the mobile phase composition

HVA and HIAA eluted at the dead volume when the mobile phases were buffered at pH 7. This is due the presence of a carboxylic group, which is negatively charged at a pH above 6. Owing to the protonation of this group, the retention factor increases as the pH decreases from 5 to 3. For this reason the pH was set at 3, where these compounds are in molecular form.

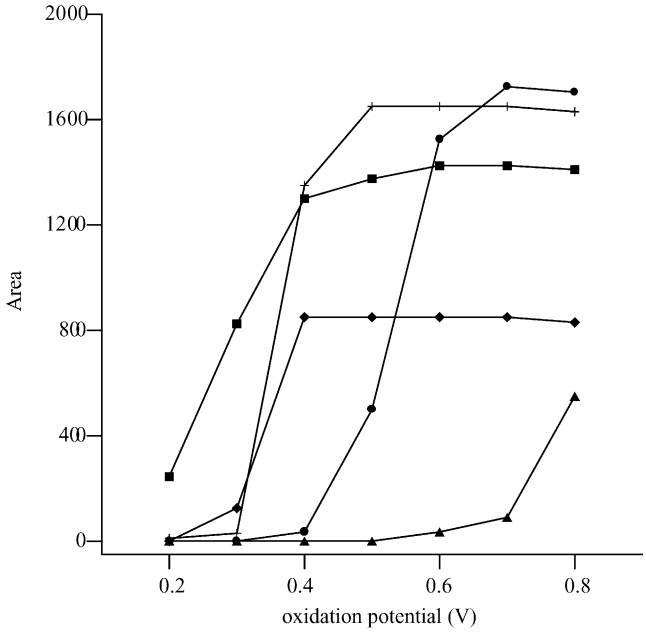

Fig. 2. Oxidation curves for the compounds studied using electrochemical detection. The hydrodynamic voltammograms were obtained by plotting the relative peak areas of each standard as a function of the potential (*V*). Dopamine (■), serotonin (♦), homovalinic acid [HVA] (●), hydroxyindoleacetic acid [HIAA] (+) and tyramine (▲).

In MLC, peak efficiencies decrease at increasing concentrations of surfactant, and increase at larger concentrations of modifier. This behavior was not observed for several catecholamines by other authors [34,35]. A decrease in efficiency is observed when an alcohol is added. This can be explained because the solubility and transfer rate of the highly hydrophilic BAs through the continuous aqueous pseudo-phase probably decreases when the alcohol concentration increases. Fig. 3a depicts the diminution of the efficiency when the concentration of the surfactant increases. As shown in Fig. 3b, dopamine and tyramine were strongly retained at low concentrations of SDS, and as the concentration of the surfactant increased a reduction in the retention times was observed. This phenomenon can be explained by the fact that the BAs are strongly bounded to the modified stationary phase and to the micelles.

Propanol is a modifier that is usually added to micellar mobile phases to increase the efficiency of the chromatographic peaks and to decrease and control retention. Although the addition of this alcohol reduced the retention factor of dopamine, serotonin and tyramine, the peaks of HVA and HIAA were overlapped. Therefore, to facilitate the separation of these two compounds, the following experiments were carried out at pH 3 without the addition of an organic modifier.

The five compounds were easily resolved using only SDS, in the 0.025–0.15 M range. k and N were higher at the lower concentration of SDS used (k = 3.8 and N = 930, for the less retained compound HIAA, and k = 63.24 and N = 3700 for the most retained compound tyramine), which was drastically

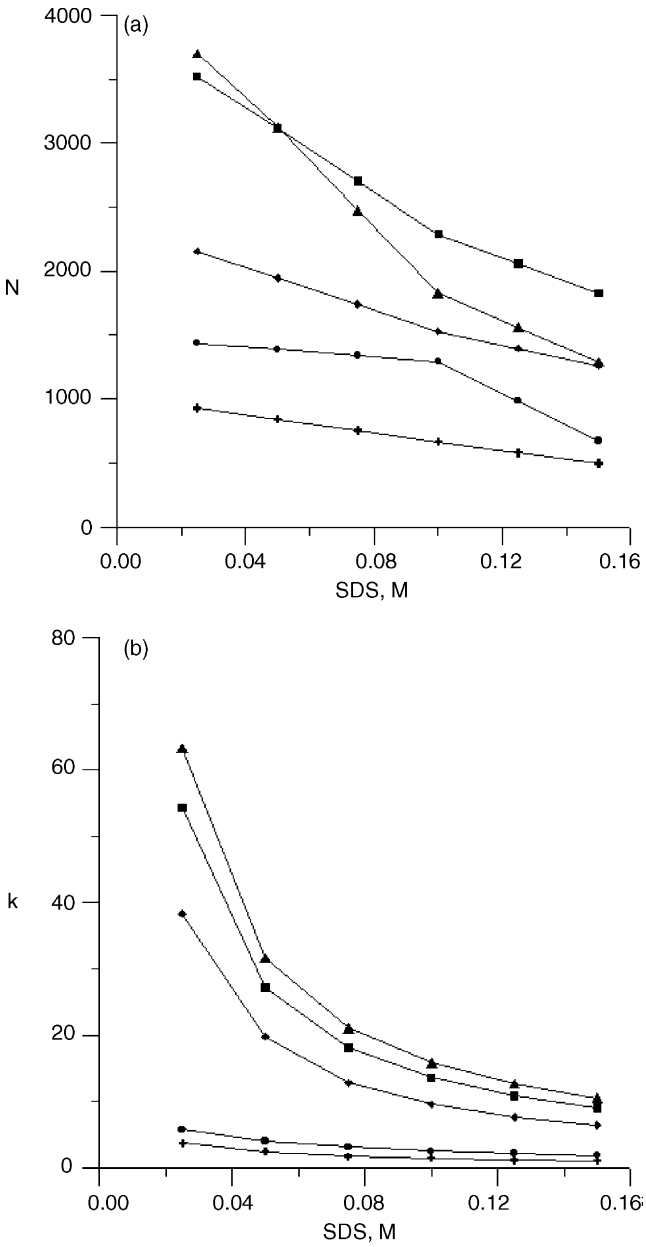

Fig. 3. Effect of the concentration of SDS on the efficiencies, N (a) and retention factors, k (b) for the biogenic amines, using mobile phases buffered at pH 3. See Fig. 2 for symbol assignation.

reduced using 0.15 M SDS (k = 0.99 and N = 500, and k = 10.54 and N = 1290 for HIAA and tyramine, respectively).

Since the analytes were never overlapped in the whole space of SDS and to diminish analysis time, the mobile phase with the highest elution strength was finally chosen to carry out the analysis (0.15 M SDS). The total chromatographic analysis time was 12 min.

3.3. Figures of merit

Calibration curves were constructed for each BA in aqueous solutions and spiked serum samples, using the measured areas of the chromatographic peaks at nine increasing concentrations in the 0.025–5 μg/mL range. The slopes of the calibration curves in the absence and presence of serum were similar, the intercepts were statistically zero for most cases, and regression coefficients were always $r > 0.9999$. Therefore, no matrix effect existed in the serum samples. The slopes, intercepts and regression coefficients of the calibration curves are given in Table 1.

The limits of detection (LODs) were calculated from the standard deviation of 10-fold injections of serum samples (3s criterion, which corresponds to a signal equal to three times the standard deviation of the background noise), spiked with the BAs at the lowest concentration of the calibration curve. The LODs were lower when electrochemical detection (ED) was used (Table 1). The LODs in serum were between 0.17 ng/mL for HIAA and 1.8 ng/mL for HVA using ED and between 3.2 and 65 ng/mL for serotonin and HIAA, respectively, using UV. The LODs obtained were similar to those usually reported in the literature, and allowed for the detection and quantification of the biogenic amines in serum, taking into account that the serum samples were injected without any previous treatment than dilution. The serum LODs were similar to those obtained from the injection of aqueous solutions of the BAs. The normal basal levels of the studied BAs in plasma from healthy subjects are 0.5–44 ng/mL for serotonin, 1.94 ng/mL for HIAA [36], 10–120 ng/mL for dopamine [37], and 5.4–10.9 ng/mL for HVA [38]. The plasma concentration of tyramine depends on the amount of this BA ingested from foodstuff.

3.4. Analysis of spiked serum samples

MLC is able to carry out analysis with minimum sample pretreatment. Generally, UV detection is not suitable for most hydrophilic compounds that elute at the dead time together with the protein band, thus interfering in the analysis. However, this problem is greatly reduced by using ED, as it depends on the oxidation of the substance eluting at the dead time. The interference from endogenous compounds that also appear when working with biological samples using UV detection is eliminated with ED working at lower potentials.

Moreover, the injection of a large number of serum samples can produce damage to the packing material and consequently shorten the column life, which can lead to the need for frequent regeneration of the stationary phase. It was therefore decided that the analysis of serum samples should be carried out after their dilution. For all the biogenic amines studied, the sensitivity achieved after dilution in a 1:5 factor was adequate for their detection in serum. In these conditions, retention times were unchanged at least after 500 injections into the chromatographic system.

3.4.1. Comparison between UV and ED

A comparison between two detection modes, UV and ED, is presented. For all the mobile phases used, the efficiency factors were similar using UV or ED detectors, but LODs

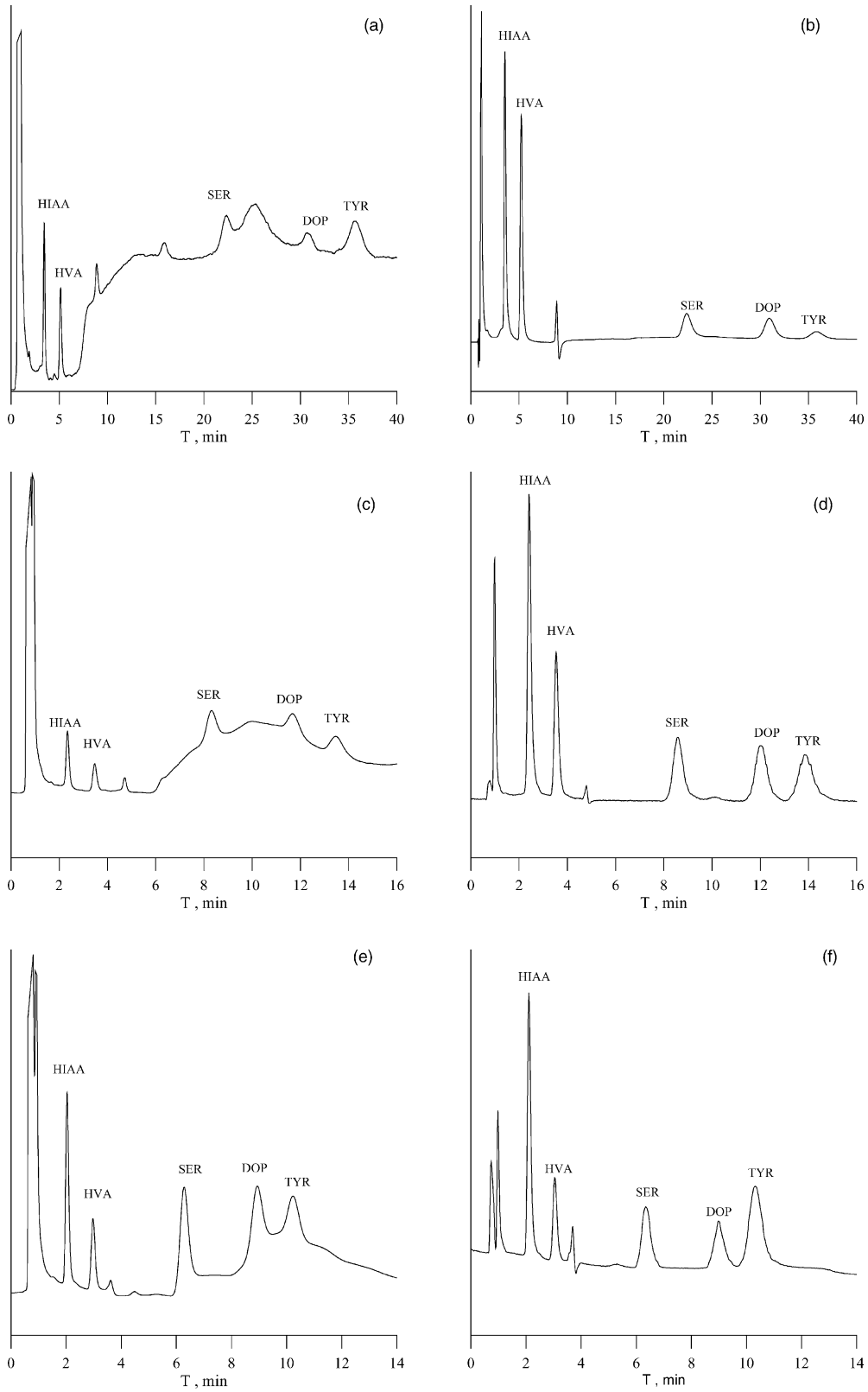

Fig. 4. Chromatograms of the spiked serum samples, at three different concentrations of SDS using UV (left side) or electrochemical (right side) detection. SDS concentration: (a) and (b) 0.05 M; (c) and (d) 0.10 M; (e) and (f) 0.15 M. Drug concentration: 0.5 μg/mL for HIAA, HVA and DOP, and 1 μg/mL for SER and TYR.

Table 1
Parameters of the calibration curves (slope, intercept and regression coefficient, *r*), limits of detection (LOD, ng/mL) and quantification (LOQ, ng/mL) for the drugs studied, using electrochemical and UV detection

Compound	Electrochemical detection					UV detection				
	Slope	Intercept	*r*	LOD	LOQ	Slope	Intercept	*r*	LOD	LOQ
HIAA	8.03	29.29	0.99995	0.17	0.47	0.2	0.046	0.9999	65	73
Serotonin	1.41	1.71	0.99998	0.42	0.79	0.27	0.022	0.99999	3.2	5.5
Dopamine	2.51	7.15	0.99993	0.9	1.23	0.06	0.006	0.99999	6.6	8.4
HVA	0.53	0.33	0.99997	1.8	2.01	0.18	0.039	0.99982	47	55
Tyramine	1.07	2.65	0.99998	0.8	1.16	0.05	0.091	0.99997	12	15

were better when ED was employed (Table 1). It was also observed that the background signal of serum samples, due to the proteins (wide band at the head of the chromatograms) and several endogenous compounds, can seriously affect UV detection as compared to ED. It can be also observed that the chromatogram baselines obtained with ED (Fig. 4b, d and f) are far better than those obtained with UV detection (Fig. 4a, c and e) for the same test solution. As can be seen the LODs obtained by ED are good enough to monitor de BAs in plasma samples.

3.4.2. Repeatability and intermediate precision

The intra- and inter-day assay precision could be not very appreciable when using ED. As the detection is based on a chemical transformation, there are chances of polluting the detector with continuous injection. The area may vary with the first and the last injection. A proper and regular cleaning protocol of the electrode eliminate this problem. Fresh daily prepared buffer solutions also contributed to the good repeatability. In general, an enhancement in the peak area of the compounds was observed when working with fresh solutions of the BAs. It should be noted that the stability of the biogenic amines is not very good since most of them are easily oxidizable and light sensitive, and HIAA and serotonin are also sensitive to the temperature.

Repeatability values were calculated by measuring the peak areas of the compounds obtained by injection of series of 10 replicates of spiked serum samples at three concentrations of the biogenic amines. The variation coefficients in the intra-day assay were always below 1.2% (Table 2). The inter-day repeatability was also determined. The variation coefficients of the peak areas in samples injected on five different days were in the 0.25–1.50% range.

Table 2
Intra- and inter-day assay (CV (%); $n = 10$) values for the determination of the drugs studied in serum samples

Compound	Repeatability			Intermediate precision		
	c_1	c_2	c_3	c_1	c_2	c_3
HIAA	0.75	0.37	0.25	0.84	0.42	0.25
Serotonin	0.49	0.42	0.54	0.58	0.64	0.74
Dopamine	0.63	0.53	0.51	0.72	0.71	0.69
HVA	1.16	0.94	0.74	1.5	1.26	1.03
Tyramine	0.81	0.74	0.64	0.91	0.89	0.87

c_1 = 1.125 μg/mL, c_2 = 2.5 μg/mL, c_3 = 5 μg/mL.

The method described above has an excellent sensitivity for serum samples. To prove the usefulness of this procedure, blank serum samples were spiked with known amounts of each BA and then injected in the chromatographic system. Table 3 shows the good recoveries obtained (91–106%, UV and 98–102%, ED) for each BA at three different concentrations.

Finally, the method was applied to the determination human blood. A blood sample was collected from a healthy subject, and centrifuged immediately to separate plasma. Serotonin and HIAA were detected. Dopamine is one of the transmitters in the brain that regulates mood, but it was not found. However, its metabolite was detected (HVA). Fig. 5 shows the chromatogram of the human plasma. The concentrations of the BAs found were: 1.80 ng/mL for HIAA, 2.8 ng/mL for HVA and 5.19 ng/mL for serotonin.

It can be concluded that the proposed procedure can be easily used for the determination of biogenic amines in serum with a chromatographic analysis time below 12 min using a mobile phase of 0.15 M SDS at pH 3. The procedure is sensitive enough to verify the concentration of the five substances in monitoring dietary biogenic amines and in studies

Table 3
Determination of the drugs in spiked serum samples ($n = 10$) at three different concentrations (μg/mL) using UV and electrochemical detection

Compound	Added	UV		ED	
		Found	R.S.D.[a] (%)	Found	R.S.D.[a] (%)
HIAA	1	1.04	0.9	1	0.5
	5	4.95	0.7	4.99	0.7
	10	9.9	0.4	9.99	0.2
Serotonin	1	0.91	1.2	0.98	0.9
	5	4.92	0.6	4.97	1
	10	9.91	0.9	10.02	0.4
Dopamine	1	0.96	1.6	0.98	1.1
	5	4.96	1	5.1	1.2
	10	10.13	0.9	10.01	0.6
HVA	1	0.97	0.6	1.02	0.4
	5	4.97	0.6	5.03	0.3
	10	10	0.2	10.01	0.4
Tyramine	1	0.93	1.1	0.99	0.6
	5	5.3	0.5	5.02	0.4
	10	9.8	0.7	9.94	0.2

[a] R.S.D.: residual standard deviation.

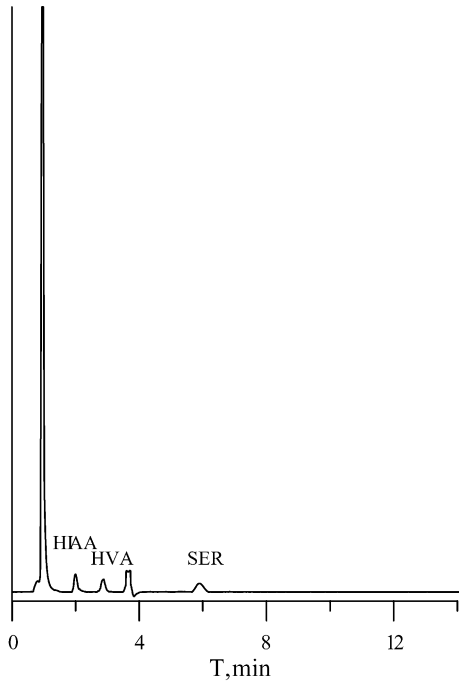

Fig. 5. Chromatogram of human serum sample using electrochemical detection. Mobile phase: 0.15 M SDS buffered at pH 3.

conducted in order to diagnose pheochromocytoma, with LODs similar to those usually reported in the literature, taking into account that the serum sample was injected without any previous treatment than filtration. It must be highlighted that the mobile phases used in this work did not require the addition of an organic solvent to achieve adequate retention of the BAs. In most micellar chromatographic procedures reported in the literature, a small amount of a short-chain alcohol must be added to accelerate and control the elution of the solutes. A decrease in the organic solvent content has been considered an interesting advantage of micellar mobile phases over aqueous-organic eluents and the procedure described in this work has the advantage of completely eliminating the polluting and flammable solvents.

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