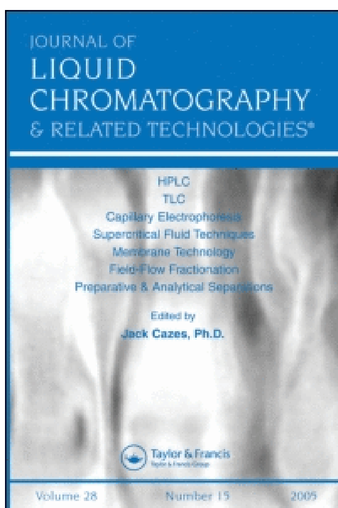
This article was downloaded by:
On: *23 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Direct Injection Analysis of Epinephrine, Norepinephrine, and their Naturally Occurring Derivatives in Serum by Micellar Liquid Chromatography with Electrochemical Detection

Devasish Bose[a]; Abhilasha Durgbanshi[a]; Samuel Carda-Broch[a]; Mayte Gil-Agustí[a]; Maria-Elisa Capella-Peiró[a]; Josep Esteve-Romero[a]
[a] Departament de Ciències Experimentals, Àrea de Química Analítica, Universitat Jaume I, Campus de Riu Sec, casló, Spain

To cite this Article Bose, Devasish , Durgbanshi, Abhilasha , Carda-Broch, Samuel , Gil-Agustí, Mayte , Capella-Peiró, Maria-Elisa and Esteve-Romero, Josep(2005) 'Direct Injection Analysis of Epinephrine, Norepinephrine, and their Naturally Occurring Derivatives in Serum by Micellar Liquid Chromatography with Electrochemical Detection', Journal of Liquid Chromatography & Related Technologies, 28: 20, 3265 — 3281
To link to this Article: DOI: 10.1080/10826070500330968
URL: http://dx.doi.org/10.1080/10826070500330968

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

Journal of Liquid Chromatography & Related Technologies®, 28: 3265–3281, 2005
Copyright © Taylor & Francis, Inc.
ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070500330968

Direct Injection Analysis of Epinephrine, Norepinephrine, and their Naturally Occurring Derivatives in Serum by Micellar Liquid Chromatography with Electrochemical Detection

Devasish Bose*, Abhilasha Durgbanshi*, Samuel Carda-Broch, Mayte Gil-Agustí, Maria-Elisa Capella-Peiró, and Josep Esteve-Romero
Departament de Ciències Experimentals, Àrea de Química Analítica, Universitat Jaume I, Campus de Riu Sec, Castelló, Spain

Downloaded At: 18:34 23 January 2011

Abstract: The plasma level determination of catecholamines and their metabolites is necessary in studies aimed at evaluating neuroendocrine disorders. A micellar liquid chromatographic procedure was developed for the determination of epinephrine, norepinephrine, and their naturally occurring derivatives, metanephrine and nor-metanephrine, in serum samples using direct injection. The optimisation studies were performed in a C_{18} column, using solutions containing sodium dodecyl sulphate (SDS) modified with propanol, butanol, or pentanol as mobile phases. The method proposed for the determination of these catecholamines used a hybrid micellar mobile phase of 0.075 M SDS–1.6% butanol–0.01 M phosphate buffer (pH 7) at 25°C, and electrochemical detection. The serum samples were injected directly without any pretreatment and eluted in 14 min, in accordance with their relative polarities as indicated by their octanol–water partition coefficients. Calibration was linear in the 0.5–50 ng mL^{-1} range with $r^2 > 0.999$. The limits of quantification (pg mL^{-1}) in serum samples were within the 2.7–17 range. Repeatability and intermediate precision were tested for four different concentrations of the drugs, and the residual

*On a research visit from the Department of Criminology and Forensic Science, Dr. Hari Singh, Gour University, Sagar, India.
Address correspondence to Josep Esteve-Romero, Departament de Ciències Experimentals, Àrea de Química Analítica, Universitat Jaume I, Campus de Riu Sec, Avda Sos Baynat s/n, 12080, Castelló, Spain. E-mail: estevej@exp.uji.es

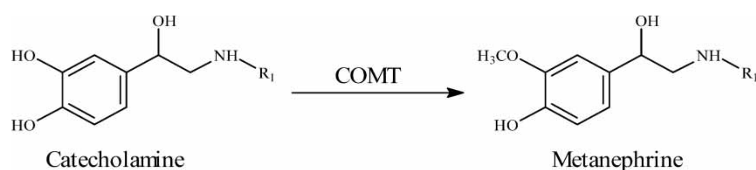
standard deviation values were below 2% for most of the assays. A study using UV detection is presented for comparison purposes.

Keywords: Epinephrin, Norepinephrine, Electrochemical detection, Serum

INTRODUCTION

Epinephrine and norepinephrine are the main endogenous catecholamines, which can act as hormones and/or neurotransmitters in several physiological and pathological situations related to both the autonomic and central nervous systems. Catecholamine drugs are also used to treat hypertension, bronchial asthma, and organic heart disease, and they are employed in cardiac surgery and myocardial infarction.[1,2] Levels of the catecholamines, epinephrine and norepinephrine and their 3-*O*-methylated metabolites (metanephrine and normetanephrine, see Figure 1) in biological fluids, are of clinical interest in fields such as the diagnosis of altered functioning of catecholamine-synthesising tissues, assessment of disease severity and prognosis, and the study of adaptive responses to stress and pharmacotherapy.[3] Recently, the measurement of plasma free metanephrine and normetanephrine has been claimed to be clinically more sensitive than urinary free catecholamines and metanephrines.[4]

Downloaded At: 18:34 23 January 2011



	Compound	R_1	log *P* [a]	pK_a [b]
Catecholamines	Epinephrine	CH_3	-1.37	8.59
	Norepinephrine	H	-1.24	8.58
Metanephrines	Metanephrine	CH_3	-0.64	NF
	Normetanephrine	H	-0.54	8.82

[a] From reference [30]

[b] From reference [31]

NF: Data not found

Figure 1. Metabolic pathway of catecholamines studied. COMT = catechol-*o*-methyltransferase.

The plasma level determination of catecholamines and their metabolites is necessary in studies aimed at evaluating neuroendocrine disorders and the role of the autonomic nervous system in several physiological and pathological situations and, on the other hand, catecholamines are major physiological markers of human stress.[5–8] The accurate assay of catecholamines in response to experimental psychological stress could be useful to investigate the possible changes associated with aggressiveness and anxiety.[9]

Nowadays, high performance liquid chromatography (HPLC) is the most commonly used technique for measuring these catecholamines and their metabolites in biological samples. The detection methods prevalently found in most research works are electrochemical,[8,10–13] fluorimetric,[14,15] and chemiluminescence.[16,17] The introduction of electrochemical detection (HPLC-ED) has provided a new tool to determine the levels of these compounds.[18,19] Catecholamines and their metabolites can be detected by their reversible oxidation at a carbon-based electrode. The amounts of neuronal amines to be determined in plasma are in the submicroanalysis range, i.e., a few parts per billion. This is a difficult analytical problem, especially if one takes into account the complex matrix from which they have to be extracted. Despite the large number of papers published on this subject, there are still problems associated with the plasma sample clean-up procedure. Due to the complexity of biological matrixes, such as urine or plasma, very effective and intensive clean-up steps are required. Human plasma catecholamines are usually purified by adsorption on alumina[8,20] or on boric gel[21] or by extraction with organic solvents.[22] However, these methods are not sensitive enough and, furthermore, they often do not give high extraction yields.

Micellar liquid chromatography (MLC), which uses mobile phases of surfactants above the critical micellar concentration, has proven to be a useful technique in the determination of diverse groups of drugs in biological fluids by direct injection of the sample.[23–28] MLC provides a solution to the direct injection of physiological samples by solubilizing the protein components, and coating the analytical column with surfactant monomers to avoid clogging.[29] One of the major advantages of MLC is its capacity to describe the retention behaviour of compounds eluted with hybrid micellar mobile phases of surfactant and organic modifiers with a high degree of accuracy. This description allows the simple selection of the optimum composition of the mobile phase.

The aim of this work is to propose a rapid and sensitive method for the determination of epinephrine, norepinephrine, metanephrine, and normetanephrine in serum samples using electrochemical detection and direct injection. The chromatographic behaviour of the highly hydrophilic catecholamines eluted with pure and hybrid micellar mobile phases is also studied. A comparison between two detection modes, UV and ED, is presented.

Downloaded At: 18:34 23 January 2011

EXPERIMENTAL

Reagents

Sodium dodecyl sulphate (SDS, 99% purity, Merck, Darmstadt, Germany), 1-pentanol, 1-butanol, 1-propanol (Scharlab, Barcelona, Spain), sodium dihydrogenphosphate, potassium chloride (Panreac, Barcelona), HCl, NaOH (Probus, Badalona, Spain) were used to prepare the mobile phases. Methanol (Scharlab) was employed to clean the column.

Epinephrine, norepinephrine, metanephrine, and normetanephrine were purchased from Sigma (St. Louis, MO, USA). Stock solutions containing 10 mg L^{-1} of the catecholamines were prepared in distilled deionised water (Barnstead, Sybron, Boston, MA, USA), and conveniently diluted for analysis.

Downloaded At: 18:34 23 January 2011

Instrumentation

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 chromatographic system (model HP 1100, Palo Alto, CA, USA), equipped with a quaternary pump, an autosampler, a thermostated column compartment, a UV-Vis, and an electrochemical detector (model HP 1049A, Palo Alto, CA, USA) was used, and a Kromasil C_{18} column (5 μm particle size, 120 mm × 4.6 mm i.d.) (Scharlab) was also employed. Injection of the solutions into the chromatograph was performed through a Rheodyne valve (Cotati, CA, USA). The dead time was determined as the mean value of the first significant deviation of the base line in the chromatograms. The signal was acquired by a PC connected to the chromatograph through an HP Chemstation.

Serum Sample Preparation

Human blood samples were collected using a DB SST Tube (BD Vacutainer Systems, Plymouth, UK) and centrifuged for 5 min at 3000 rpm at 4°C. The human analyses were performed with 1 mL of the serum samples, which was diluted in a ratio of 1 : 5 with the mobile phase before injection. The aqueous and serum solutions of the catecholamines were injected into the chromatographic system without any pretreatment except filtration, which was carried out directly into the autosampler vials through 0.45 μm Nylon membranes (Micron Separations, Westboro, MA, USA). The optimisation of the procedure was performed with spiked serum samples containing accurately known amounts of catecholamines.

HPLC conditions

A micellar mobile phase composed of 0.75 M SDS, 1.6% butanol, and 0.01 M NaH_2PO_4 was adjusted to pH 7 and then filtered through 0.45 μm Nylon membranes (Micron Separations). The pH was measured after the addition of the SDS and before the addition of the alcohol. No effect was observed in the performance of the pH electrodes. The column temperature was set at $25 \pm 0.2°C$. The flow rate and injection volume were 1.0 mL min^{-1} and 20 μL, respectively. Monitoring was performed at 280 nm for UV detection and a potential of 0.7 V was applied across the electrodes of the electrochemical detector.

RESULTS AND DISCUSSION

Optimisation of the Oxidation Potential

In order to establish the most adequate oxidation potential, which gives the maximum peak area, for the detection of the four studied substances, hydrodynamic voltammograms were plotted (Figure 2). The potential applied was varied between +0.1 and +0.8 V, in 0.1 V steps. At each voltage, ten injections of each substance were made and the peak area measured. Results indicated that catecholamines were oxidised at potentials higher than 0.2 V, whereas their metabolites were oxidised at potentials above 0.4 V. The sensitivity of the analytes increased in parallel to the applied potential up to 0.6–0.7 V range. On the other hand, the use of potentials higher than 0.7 V gave rise to a loss of reproducibility, possibly due to contamination of the electrode surface by the oxidation products. In this work, optimum oxidation potential under the criteria of maximum area, minimum voltage, occurs at 0.7 V.

pH Selection

Figure 1 indicates the octanol-water partition coefficients and protonation constants of the catecholamines in aqueous solution.[30,31] We did not find the pK_a of metanephrine in the literature, but it should be similar to the other compounds studied because their structure and functional groups are very similar. The micelles of the anionic surfactant increase the stability of the protonated species of the drugs and, consequently, their protonation constants. Since the protonation constants of the catecholamines and their metabolites studied are above 8.5, they are protonated in the working pH range of the column ($2.5 < pH < 7.5$) used. Thus, the retention of the drugs did not vary in the working pH range using mobile phases of SDS, SDS-propanol, SDS-butanol, or SDS-pentanol. In addition, pH 7 is more

Downloaded At: 18:34 23 January 2011

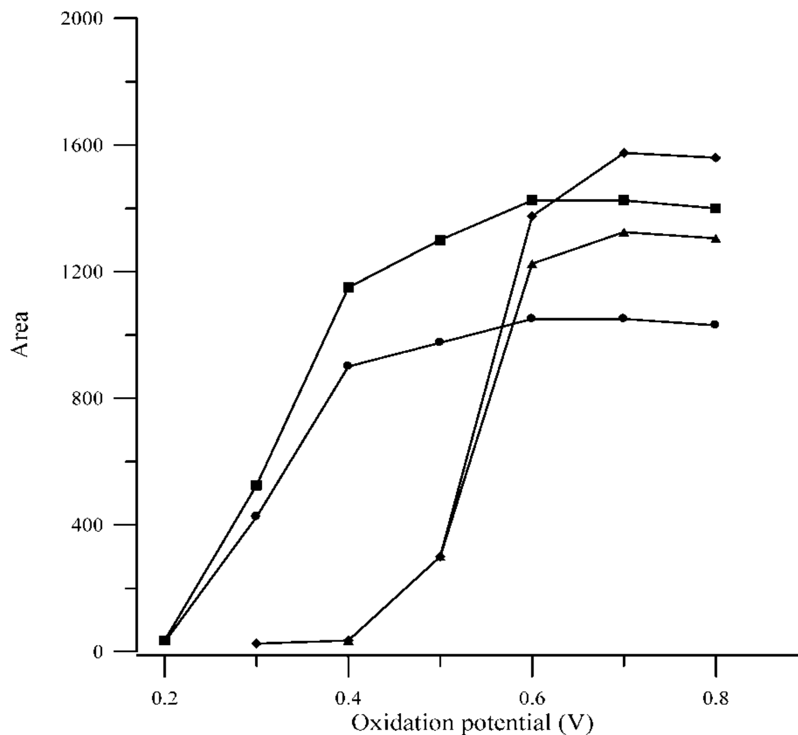


Figure 2. Oxidation curves for the compounds studied using electrochemical detection. Hydrodynamic voltammograms were obtained by plotting the relative peak areas of each standard as a function of the potential (V). Epinephrine (■), norepinephrine (●), metanephrine (◆), and normetanephrine (▲).

Downloaded At: 18:34 23 January 2011

suitable for the preservation of the column than an acidic media. Thus, the pH was set at 7 for the subsequent trials.

Effect of SDS Concentration on Retention Behaviour and Efficiency

In MLC, the mobile phase consists of an aqueous solution of surfactant at a concentration above the critical micellar concentration, in contact with an alkyl bonded stationary phase. The adsorbed surfactant layer affects the surface of the stationary phase (i) by possibly changing its charge density, and (ii) by modifying the stationary phase mobile phase interfacial tension. A layer of ionic surfactant molecules adsorbed on a stationary phase creates a charge density at the silica surface. The retention of solutes of opposite charge, as for catecholamines, is dramatically increased. Therefore, the retention of catecholamines in a C_{18} column with pure micellar eluents (without modifier) was high (>30 min). The high values of retention factors

and the high eluent strength of SDS indicated that catecholamines were strongly bound to both the modified stationary phase and the micelles. Although the retention decreased rapidly when the SDS concentration was increased, the separation of the catecholamines was not accomplished because the chromatographic peaks of metanephrine and normetanephrine were overlapped throughout all the compositions tested. On the other hand, the use of pure micellar eluents produces experimental problems like the formation of bubbles, foam, and changes in the pressure of the chromatograph. The elution order of the compounds was in accordance with their hydrophobicity (octanol water partition coefficient, Figure 1), and thus eluted in the following order: epinephrine, norepinephrine, metanephrine, and normetanephrine. Efficiencies also decreased at increasing concentration of SDS. The efficiencies of epinephrine, norepinephrine, metanephrine, and normetanephrine were 3100, 2800, 2800, and 3100, respectively, at 0.05 M SDS, while they decreased to 1600, 1900, 1800, and 2000, respectively, when the concentration of SDS was increased to 0.15 M.

Downloaded At: 18:34 23 January 2011

Effect of Organic Modifier on Retention Factor and Efficiency

The addition of a small amount of an organic solvent was convenient to decrease the retention times. Micellar eluents composed solely of surfactants are generally weak and suffer from poor efficiency. The reduced efficiency with the micellar mobile phases may be attributed to the small rate constant for solute exit from the micellar aggregates. The problem of efficiency can be overcome by the addition of an alcohol to the micellar mobile phase, as this will increase the solute mass transfer kinetics between the stationary and aqueous phase by increasing the solute micelle exit rate constant, as well as decreasing the viscosity of the stationary phase and the amount of adsorbed surfactant. Thus, when an alcohol is added to the pure micellar mobile phases, the efficiency increases and the retention time decreases.

However, the opposite behaviour was observed for catecholamines as far as efficiency is concerned. The loss of efficiency caused by the addition of alcohol when amines are eluted with an anionic micellar phase has already been observed by other authors.[32] It is assumed that mass transfer takes place by two mechanisms, that is, by direct transfer from the micelles to the stationary phase, which is opposed by the electrostatic repulsion between them, and by transfer through the continuous aqueous pseudo-phase. When alcohol is present, the polarity of the continuous phase decreases, thus increasing the transfer rate of hydrophobic solutes through it. However, the solubility and transfer rate of the highly hydrophilic catecholamines through the continuous aqueous pseudo phase probably decreased when the alcohol concentration increased, which could explain the decrease in efficiency.

The use of hybrid micellar mobile phases at varying concentrations of surfactant and modifier (such as propanol, butanol, or pentanol) produces changes

in the retention factors (k), efficiencies (N), and asymmetries (B/A) of the chromatographic peaks. Suitable control of the concentrations of surfactant and modifier is, therefore, necessary to achieve chromatograms showing good resolution and sufficient elution strength.

The optimum mobile phase for the separation of the catecholamines was obtained through the application of an interpretive procedure, which requires the retention data and peak shape parameters of the chromatographic peaks eluted in selected mobile phases of surfactant and modifier, adequately distributed in the variable (surfactant and modifier concentrations) space.[33,34] In this study, serum samples spiked with the drugs were injected into the chromatographic system and eluted with seven mobile phases, in a design with four mobile phases located in the corners of a rectangle, one at its centre, and another two inside it (SDS (M)-propanol (%, v/v): 0.05–2.5, 0.05–12.5, 0.15–2.5, 0.15–12.5, 0.1–7.5, 0.1–10, 0.1–5); SDS (M)-butanol (%, v/v): 0.05–1, 0.05–7, 0.15–1, 0.15–7, 0.1–4, 0.1–2.5, 0.15–5; and SDS (M)-pentanol (%, v/v) 0.05–2, 0.05–6, 0.15–2, 0.15–6, 0.1–4, 0.1–3, 0.1–5. All contained phosphate buffer at pH 7). The retention behaviour of the drugs was modelled according to:[29]

$$k = \frac{K_{AS} \dfrac{1 + K_{SD}\varphi}{1 + K_{AD}\varphi}}{1 + K_{AM} \dfrac{1 + K_{MD}\varphi}{1 + K_{AD}\varphi}[M]} \tag{1}$$

where $[M]$ and φ are the concentrations of surfactant and modifier, K_{AS} and K_{AM} correspond to the equilibrium between solute in bulk water and stationary phase or micelle, respectively; K_{AD}, K_{SD}, and K_{MD} measure the relative variation in the concentration of solute in bulk water, stationary phase, and micelles due to the presence of modifier, as referred to a pure micellar solution (without modifier).

The accurate prediction of the retention according to eq. (1) allowed the application of an interpretive procedure to predict the optimum mobile phase, following a criterion that uses the overlapping fractions.[35] Incorporation of the peak shape in the optimisation procedure improves the results. The reliable simulation of the peak shape for any mobile phase of the variable space was carried out with an asymmetrical Gaussian function where the standard deviation is a first-degree polynomial function.[34] Using eq. (1) and the mathematical treatment described here, the relative global error in the prediction of retention factors was below 5% for all drugs studied.

Three optimisation procedures were carried out using the alcohols propanol, butanol, and pentanol as modifiers in each case. The analysis time of the catecholamines decreased when propanol was replaced by butanol, and then by pentanol. On the other hand, the efficiencies obtained with butanol were the highest for all the compounds compared to those obtained using propanol or pentanol (e.g., for epinephrine the efficiencies were 1900, 2300, and 1400

Downloaded At: 18:34 23 January 2011

when eluted with the mobile phases 0.1 M SDS and the modifiers propanol (7.5%, v/v), butanol (7%, v/v) and pentanol (6%, v/v), respectively). After the application of the optimisation procedure, results indicated that the resolution of the catecholamines in SDS-propanol and SDS-butanol was similar (0.989), but in SDS-pentanol the epinephrine-norepinephrine and metanephrine-normetanephrine couples are not separated from the base-line, resulting in low resolution and, on the other hand, the epinephrine-norepinephrine couple appears near the zone of the serum protein band. Mobile phases with propanol show higher retention factors for all the substances and, thus, the highest analysis time. Therefore, butanol was chosen to carry out the optimisation procedure under the criteria of good resolution, minimum analysis time.

After checking the chromatographic behaviour of the catecholamines studied in all the optimisation space, and considering the optimisation criteria (maximum resolution, maximum efficiency, minimum analysis time), a mobile phase containing SDS 0.075 M with 1.6% butanol (v/v) and 0.01 M monosodium dihydrogenphosphate at pH 7 was chosen as the optimum for separation of the four catecholamines. In this mobile phase, the retention times (min) for the catecholamines were: 8.2 for epinephrine, 8.8 for norepinephrine, 10.4 for metanephrine, and 12.2 for normetanephrine. The prediction errors obtained with the model and corresponding experimental designs are below 1.5%.

Downloaded At: 18:34 23 January 2011

Figures of Merit

Calibration curves were constructed for the catecholamines using the areas of the chromatographic peaks measured at eight increasing concentrations. The concentrations were in the 0.5–50 ng mL^{-1} range for electrochemical detection, while for UV it was in the 50–500 ng mL^{-1} range. The calibration curves were obtained for aqueous solution and spiked serum samples. The calibration parameters (slope and intercept) obtained in both, were statistically equal, meaning that the serum does not cause a matrix effect or interference in the method. The slopes and intercepts of the calibration curves with electrochemical detection were higher than those with UV detection, and the determination coefficients were usually $r^2 > 0.999$ (Table 1). Therefore, no matrix effect existed in the serum samples when the MLC procedure coupled with electrochemical detection was used.

Table 1 shows the limits of quantification (LOQs, 10s criterion, which corresponds to a signal equal to ten times the standard deviation of the background noise) evaluated by injection of series of 10 solutions containing the catecholamines at the lowest concentration of the calibration curve in serum. The LOQs in serum were between 2.7–17 pg mL^{-1} with ED, while with UV higher, LOQs in the range of 7.4–11 ng mL^{-1} were obtained. The ED values of the LOQs allowed the detection and quantification of the catecholamines in serum with the method proposed in this work, taking into account that the serum samples were injected without any previous treatment.

Downloaded At: 18:34 23 January 2011

Table 1. Parameters of the calibration curves (slope, intercept and determination coefficient, r^2), and limits of quantification (LOQ) for the drugs studied, using electrochemical and UV detection

	Electrochemical detection				UV detection			
Compound	Slope	Intercept	r^2	LOQ (pg mL^{-1})	Slope	Intercept	r^2	LOQ (ng mL^{-1})
Epinephrine	1.03	0.77	0.99998	4.6	0.15	0.025	0.9999	7.9
Norepinephrine	8.48	0.84	0.998	7.3	0.49	0.276	0.997	7.4
Metanephrine	3.63	1.15	0.99996	2.7	1.84	0.801	0.9994	10
Normetanephrine	1.48	1.52	0.9996	17	0.90	0.190	0.9997	11

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

	Intra-assay precision				Inter-assay precision			
Compound	c_1	c_2	c_3	c_4	c_1	c_2	c_3	c_4
Epinephrine	0.92	0.86	0.14	0.11	1.43	1.01	0.32	0.42
Norepinephrine	1.21	0.95	0.22	0.14	1.67	1.13	0.29	0.32
Metanephrine	1.02	0.59	0.18	0.11	1.21	0.81	0.23	0.18
Normetanephrine	1.71	0.78	0.15	0.12	1.81	0.91	0.41	0.53

$c_1 = 50$ pg mL^{-1}, $c_2 = 100$ pg mL^{-1}, $c_3 = 250$ pg mL^{-1}, $c_4 = 1$ ng mL^{-1}.

Repeatability values were calculated by measuring the areas of the peaks obtained by injection of series of ten replicates of spiked serum samples at four concentrations between 50 pg mL^{-1} and 1 ng mL^{-1} (Table 2). The variation coefficient was always below 2.0%. The inter-day repeatabilities (intermediate precision; samples injected along ten different days) were also obtained, and the variation coefficients were in the 0.2–1.8% range (Table 2).

Downloaded At: 18:34 23 January 2011

Analysis of Serum Samples

The background signal of serum samples, due to the proteins (wide band at the head of the chromatograms) and several endogenous compounds (peaks at diverse retention times), can seriously affect the detection of catecholamines. In the UV detection mode, direct injection of serum samples gives a broad band at the head of the chromatogram with severe tailing, which affects the detection of compounds in a low concentration range. This obstacle is overcome by using ED, since the width of the band depends on the oxidation of the eluting compounds, which are mainly serum protein and some endogenous substances that are mainly non-oxidizable.

Moreover, the injection of a large number of serum samples can produce damage to the packing material, thus shortening the life of the column, or can make frequent regeneration of the stationary phase necessary. It was, therefore, decided to carry out the analysis of serum samples after their dilution. For all the studied compounds, the sensitivity achieved after dilution in a ratio of 1 : 5 was adequate for their detection in serum.

To determine the specificity of the assay, blank serum samples collected from healthy adult volunteers were analysed using the reported procedure. No interfering peaks from endogenous compounds appeared at the same retention times for the drugs studied when the chromatograms were inspected. Thus, the specificity of the procedure was successfully proven in this assay.

The method described above has an excellent sensitivity for serum samples. To prove the usefulness of this procedure, blank serum samples

Table 3. Determination of the drugs in spiked serum samples (n = 10) at three different concentrations (ng mL^{-1}) using UV and electrochemical detection

	UV			ED		
Compound	Added	Found	RSD[a] (%)	Added	Found	RSD (%)
Epinephrine	15	15.04	1.2	0.5	0.499	0.8
	30	30.12	0.9	1.0	1.01	0.4
	50	49.81	0.7	2.0	1.97	0.3
Norepinephrine	15	14.98	1.4	0.5	0.495	1.2
	30	30.09	0.6	1.0	0.98	0.8
	50	48.11	0.9	2.0	1.99	0.6
Metanephrine	15	15.46	1.1	0.5	0.489	0.9
	30	29.96	1.0	1.0	1.04	0.8
	50	50.11	0.8	2.0	2.04	1.3
Normetanephrine	15	14.76	1.9	0.5	0.51	1.0
	30	29.88	0.9	1.0	1.03	0.2
	50	50.01	0.2	2.0	1.98	0.4

[a]RSD = residual standard deviation.

Downloaded At: 18:34 23 January 2011

were spiked with known amounts of each catecholamine (three different concentrations) and then injected into the chromatographic system. Table 3 shows the amounts found in the spiked serum samples (recoveries were in the 96–103% range for UV and 98–104% for ED detection).

Determination of Catecholamines in Human Blood

Samples of serum from several volunteers (n = 4) under emotional stress were analysed and the concentrations of catecholamines determined. After the extraction of blood for the determination of serum catecholamines, the samples were immediately centrifuged and frozen at −80°C. The mean concentration (pg mL^{-1}) of these substances in healthy volunteers were: epinephrine 75 ± 15, norepinephrine 320 ± 20, metanephrine 40 ± 8, and normetanephrine 60 ± 5 pg mL^{-1}. These results have been obtained using an electrochemical detector set at 0.7.V LOQs, allow determination of these concentrations in real serum samples. Figure 3 depicts a chromatogram of human serum from a healthy volunteer.

For comparative purposes, serum samples were compared with those obtained with an HPLC method, using an aqueous organic mobile phase containing 15% methanol, 0.1 M citric acid, 0.1 M sodium acetate (pH 5.5), 0.4 g 1-octanesulphonic acid. The recommended potential was 780 mV. Sample pretreatment was performed by adding 25 mg of alumina to each standard, adjusting the pH to

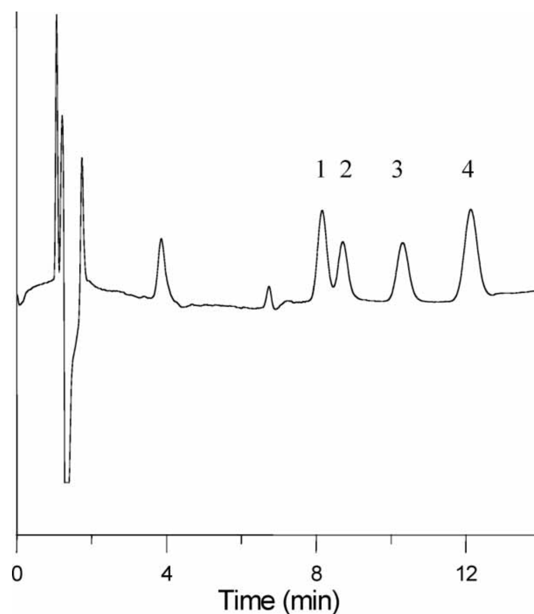


Figure 3. Chromatogram of human serum samples of catecholamines eluted in the mobile phase 0.075 M SDS 1.6% butanol with electrochemical detection. Peak assignation: Epinephrine (1), norepinephrine (2), metanephrine (3), and normetanephrine (4).

8.6 with Tris-EDTA buffer, shaking for 10 min, washing the alumina twice with water, and finally eluting the catecholamines with $HClO_4$ 100 mM. We checked the analytical accuracy of the results by obtaining the linear regression parameters of MLC versus aqueous organic HPLC data, with regression coefficients above 0.94 (n = 22). Results for each catecholamine were: MLC = 0.984 * HPLC − 0.029 (R = 0.95) for epinephrine, MLC = 0.977 * HPLC − 0.067 (R = 0.94) for norepinephrine, MLC = 0.984 * HPLC − 0.056 (R = 0.97) for metanephrine, and MLC = 0.994 * HPLC − 0.075 (R = 0.95) for normetanephrine. The MLC method gave slightly higher concentrations for catecholamines as compared to aqueous organic, although there was no substantial difference between both in the practical sense of clinical analysis.

Interference Studies

Substances tested as interferents in this work were: ampicillin, acetaminophen, amphetamine, benzodiazepines, caffeine, chlorpromazine, cimetidine, dexamethazone, ephedrine, phenothiazines, hydralazine, isoetharine, isoproterenol, labetolol, levodopa, metamphetamine, metoclopramide, 3-methoxytyramine, methyldopa, niacin, nicotine, pseudoephedrine,

Downloaded At: 18:34 23 January 2011

phenylpropanolamine, promethazine, reserpine, salsolinol, serotonin, and theophylline. These substances were spiked to the serum in concentrations of 10 ng mL^{-1} and chromatographed.

Results indicated that using the recommended mobile phase (0.075 M SDS 1.6% (v/v) butanol pH 7) and an oxidation potential of 0.7 V, most of these substances do not interfere because they need a high oxidation potential or derivatization reactions to become electrochemically detectable. Thus, theophylline needs potentials higher than +1.0 V, for caffeine they must be above +1.2 V or photolysis to be electrochemical active, cimetidine is oxidised with $Hg/HgCl_2$ at 40°C and is not oxidised on a carbon electrode, amphetamines needs derivatization, and benzodiazepines are detected at a reductive mode mercury electrode. Acetaminophen is a hydrophilic substance that appears in the dead time in spiked serum samples while using the recommended mobile phase. The other substances appear after 15 min, when all the catecholamines have been eluted.

Downloaded At: 18:34 23 January 2011

CONCLUSION

The results indicate that the MLC procedure developed here can easily be used for the determination of epinephrine, norepinephrine, metanephrine, and normetanephrine in serum samples with an analysis time below 14 min, using a mobile phase of 0.075 M SDS 1.6% butanol buffered at pH 7. The procedure is sensitive enough to monitor catecholamines in clinical samples, with adequate limits of quantification, taking into account that the serum samples were injected directly into the chromatographic system without any previous treatment.

ACKNOWLEDGMENTS

This work was funded by Project P1B2003-07 (Fundació Caixa de Castelló-Bancaixa), and GV05/124 (Conselleria d'Empresa, Universitat i Ciència de la Generalitat Valenciana). Dr. A. Durgbanshi thanks the Indian Government.

REFERENCES

1. El-Kommos, M.E.; Mohamed, F.A.; Khedr, A.S.K. Spectrophotometric determination of some catecholamine drugs using metaperiodate. J. Assoc. Anal. Chem. **1990**, *73*, 516–520.
2. Sorouraddin, M.H.; Manzoori, J.L.; Kargarzadeh, E.; Haji Shabani, A.M. Spectrophotometric determination of some catecholamine drugs using sodium bismuthate. J. Pharm. Biomed. Anal. **1998**, *18*, 877–881.

Downloaded At: 18:34 23 January 2011

3. Kagedal, B.; Goldstein, D.S. Catecholamines and their metabolites. J. Chromatogr. **1988**, *429*, 177–233.
4. Eisenhofer, G.; Lenders, J.W.M.; Linehan, W.M.; Walther, M.M.; Goldstein, D.S.; Keiser, H.R. Plasma Normetanephrine and Metanephrine for Detecting Pheochromocytoma in von Hippel–Lindau Disease and Multiple Endocrine Neoplasia Type 2. Engl. J. Med. **1999**, *340*, 1872–1879.
5. Canon, W.B. Organization for physiological processes. Physiol. Rev. **1929**, *9*, 399–431.
6. Kopin, I.J. Catecholamine metabolism: basic aspects and clinical significance. Pharmacol. Rev. **1985**, *37*, 333–364.
7. Kaye, W.H.; Gwirtsman, H.E.; George, D.T.; Ebert, M.H.; Jimerson, D.C.; Tomai, T.P.; Ghrousos, G.P.; Gold, P.W. Elevated cerebrospinal fluid levels of immunoreactive corticotropin-releasing hormone in anorexia nervosa: relation to state of nutrition, adrenal function, and intensity of depression. J. Clin. Endocrinol. Metab. **1987**, *64*, 203–208.
8. Fisher, D.H.; Fisher, L.M.; Broudy, M. Evaluation of four methods for catecholamine analysis in plasma obtained from subjects during incremental cycle ergometry. J. Liq. Chromatogr. **1995**, *18*, 3311–3327.
9. Gerra, G.; Zaimovic, A.; Giucastro, G.; Folli, F.; Maestri, D.; Tessoni, A.; Avanzini, P.; Caccavari, R.; Bernasconi, S.; Brambilla, F. Neurotransmitter-hormonal responses to psychological stress in peripubertal subjects: relationship to aggressive behavior. Life Sci. **1998**, *62*, 617–625.
10. Wang, Y.; Fice, D.S.; Yeung, P.K.F. A simple high-performance liquid chromatography assay for simultaneous determination of plasma norepinephrine, epinephrine, dopamine and 3,4-dihydroxyphenyl acetic acid. J. Pharm. Biomed. Anal. **1999**, *21*, 519–525.
11. Sabbioni, C.; Saracino, M.A.; Mandrioli, R.; Pinzauti, S.; Furlanetto, S.; Gerra, G.; Raggi, M.A. Simultaneous liquid chromatographic analysis of catecholamines and 4-hydroxy-3-methoxyphenylethylene glycol in human plasma comparison of amperometric and coulometric detection. Journal of Chromatography A **2004**, *1032*, 65–71.
12. Raggi, M.A.; Sabbioni, C.; Casamenti, G.; Gerra, G.; Calonghi, N.; Masotti, L. Determination of catecholamines in human plasma by high-performance liquid chromatography with electrochemical detection. J. Chromatogr. B **1999**, *730*, 201–211.
13. Raggi, M.A.; Sabbioni, C.; Nicoletta, G.; Mandrioli, R.; Gerra, G. Analysis of plasma catecholamines by liquid chromatography with amperometric detection using a novel SPE ion-exchange procedure. J. Sep. Sci. **2003**, *26*, 1141–1146.
14. Van der Hoorn, F.A.; Boomsma, F.; Man In't Veld, A.J.; Schalekamp, M.A. Improved measurement of urinary catecholamines by liquid-liquid extraction, derivatization and high-performance liquid chromatography with fluorimetric detection. J. Chromatogr. **1991**, *563*, 348–355.
15. Iizuka, H.; Ishige, T.; Ohta, Y.; Yajima, T. Simultaneous determination of 5-hydroxyindoles and catecholamines by HPLC with fluorometric precolumn derivatization. Adv. Exp. Med. Biol. **1999**, *467*, 821–826.
16. Takezawa, K.; Tsunoda, M.; Murayama, K.; Santa, T.; Imai, K. Automatic semi-microcolumn liquid chromatographic determination of catecholamines in rat plasma utilizing peroxyoxalate chemiluminescence reaction. Analyst **2000**, *125*, 293–296.
17. Ragab, G.H.; Nohta, H.; Zaitsu, K. Chemiluminescence determination of catecholamines in human blood plasma using 1,2-bis(3-chlorophenyl)ethylenediamine as

precolumn derivatizing reagent for liquid chromatography. Anal. Chim. Acta **2000**, *403*, 155–160.
18. Cullison, J.K.; Waraska, J.; Buttaro, D.J.; Acworth, I.N.; Bowers, M.L. Electrochemical detection of catecholamines at sub-5 fg levels by redox cycling. J. Pharm. Biomed. Anal. **1999**, *19*, 253–259.
19. Ribotta, M.J.; Rodriguez, N.; Marioli, J.M. Effect of different experimental variables on the separation of catecholamine metabolites by ion-interaction RP-HPLC. J. Liq. Chromatogr. Rel. Technol. **1998**, *21*, 1003–1021.
20. He, H.B.; Stein, C.M.; Christman, B.; Wood, A.J.J. Determination of catecholamines in sheep plasma by high-performance liquid chromatography with electrochemical detection: comparison of deoxyepinephrine and 3,4-dihydroxybenzylamine as internal standard. J. Chromatogr. B **1997**, *701*, 115–119.
21. Imai, Y.; Ito, S.; Maruta, K.; Fujita, K. Simultaneous determination of catecholamines and serotonin [5-hydroxytryptamine] by liquid chromatography, after treatment with boric acid gel. Clin. Chem. **1988**, *34*, 528–530.
22. Smedes, F.; Kraak, J.C.; Poppe, H. Simple and fast solvent-extraction system for selective and quantitative isolation of adrenaline, noradrenaline and dopamine from plasma and urine. J. Chromatogr. **1982**, *231*, 25–39.
23. Capella-Peiro, M.; Bose, D.; Martinavarro-Dominguez, A.; Gil-Agusti, M.; Esteve-Romero, J. Direct injection micellar liquid chromatographic determination of benzodiazepines in serum. J. Chromatogr. B **2002**, *780*, 241–249.
24. Capella-Peiro, M.; Martinavarro-Dominguez, A.; Gil-Agusti, M.; Esteve-Romero, J. Determination in serum of some barbiturates using micellar liquid chromatography with direct injection. Anal. Biochem. **2002**, *309*, 261–268.
25. Ruiz-Angel, M.J.; Fernandez-Lopez, P.; Murillo-Pulgarin, J.A.; Garcia-Alvarez-Coque, M.C. Control of propranolol intake by direct chromatographic detection of -naphthoxylactic acid in urine. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. **2002**, *767*, 277–283.
26. Carda-Broch, S.; Esteve-Romero, J.; Ruiz-Angel, M.J.; Garcia-Alvarez-Coque, M.C. Determination of furosemide in urine samples by direct injection in a micellar liquid chromatographic system. Analyst **2002**, *127*, 29–34.
27. Torres-Cartas, S.; Villanueva-Camañas, R.M.; Garcia-Alvarez-Coque, M.C. Determination of steroidal hormones in urine samples by micellar liquid chromatography following solid-phase extraction. J. Liq. Chromatogr. Relat. Technol. **2001**, *24*, 1089–1103.
28. Martinavarro-Dominguez, A.; Capella-Peiro, M.E.; Gil-Agusti, M.; Marcos-Tomas, J.V.; Esteve-Romero, J. Therapeutic drug monitoring of anticonvulsant drugs by micellar HPLC with direct injection of serum samples. Clin. Chem. **2002**, *48*, 1696–1702.
29. Berthod, A.; Garcia-Alvarez-Coque, M.C. Eds. *Micellar Liquid Chromatography*; Marcel Dekker, Inc.: New York, 2000.
30. Drayton, C.J. Ed. *Comprehensive Medicinal Chemistry*; Pergamon Press: Oxford, England, 1990; Vol. 6.
31. Hansch, C.; Leo, A.; Hoekman, D. *Exploring QSAR: Hydrophobic, electronic, and steric constants*; Am. Chem. Soc.: Washington, 1995.
32. Palmisano, F.; Guerrieri, A.; Zambonin, P.G.; Cataldi, T.R.I. Determination of methotrexate in untreated body fluids by micellar liquid chromatography. Anal. Chem. **1989**, *61*, 946–950.

Downloaded At: 18:34 23 January 2011

33. Garcia-Alvarez-Coque, M.C.; Torres-Lapasió, J.R.; Baeza-Baeza, J.J. Modelling of retention behaviour of solutes in micellar liquid chromatography. J. Chromatogr. A **1997**, *780*, 129–148.
34. Torres-Lapasió, J.R.; Baeza-Baeza, J.J.; Garcia-Alvarez-Coque, MC. A model for the description, simulation and deconvolution of skewed chromatographic peaks. Anal. Chem. **1997**, *69*, 3822–3831.
35. Torres-Lapasió, J.R.; Villanueva-Camañas, R.M.; Sanchis-Mallols, J.M.; Medina-Hernández, M.J.; García-Alvarez-Coque, M.C. Interpretive strategy for optimization of surfactant and alcohol concentration in micellar liquid chromatography. J. Chromatogr. A **1994**, *677*, 239–253.

Received July15, 2005
Accepted August 11, 2005
Manuscript 6687

Downloaded At: 18:34 23 January 2011