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Article

Assessing Stir Bar Sorptive Extraction and Microextraction by Packed Sorbent for Determination of Selective Serotonin Reuptake Inhibitor Antidepressants in Plasma Sample by Non-Aqueous Capillary Electrophoresis

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Duas técnicas analíticas baseadas no equilíbrio de partição, extração sortiva em barra de agitação (SBSE) e microextração por sorvente empacotado (MEPS) foram avaliadas para a determinação de antidepressivos inibidores seletivos da recaptação de serotonina (SSRI): fluoxetina, sertralina, citalopram e paroxetina, em amostra de plasma por eletroforese capilar em meio não aquoso (NACE) com detecção espectrofotométrica. Os métodos SBSE/NACE e MEPS/NACE apresentaram linearidade na faixa de concentração do limite de quantificação (LOQ) até 500 ng mL^{-1}. Os valores de LOQ variaram de 10 a 30 ng mL^{-1}, em ambas as técnicas, dependendo do fármaco. A precisão interensaio dos métodos apresentou coeficiente de variação inferior a 12%. Os resultados analíticos de validação demonstraram que os métodos são adequados para análises de antidepressivos ISRS em níveis terapêuticos e, consequentemente, para fins de monitorização terapêutica. Estes métodos reduziram os volumes de amostras biológicas, a utilização de solventes orgânicos e o número de etapas do preparo das amostras.

Two approaches based on partition equilibrium, stir bar sorptive extraction (SBSE) and microextraction by packed sorbent (MEPS) were evaluated for determination of selective serotonin reuptake inhibitor antidepressants (SSRI): fluoxetine, sertraline, citalopram and paroxetine in plasma sample by non-aqueous capillary electrophoresis (NACE) with spectrophotometric detection. The SBSE/NACE and MEPS/NACE methods showed linear range from the LOQ up to 500 ng mL^{-1}. LOQ values ranged from 10 to 30 ng mL^{-1} in both techniques, depending on the compound. The inter-assay precision of the methods presented coefficient of variation lower than 12%. According to the analytical validation results, the methods are adequate for SSRI antidepressant analyses at therapeutic levels, consequently, amenable to therapeutic drug monitoring. These methods reduced the volumes of biological samples, the use of organic solvents and the number of steps involved in sample preparation.

Keywords: antidepressant, microextraction by packed sorbent, non-aqueous capillary electrophoresis, plasma sample, stir bar sorptive extraction

Introduction

The selective serotonin reuptake inhibitors (SSRIs: paroxetine, fluoxetine, citalopram and sertraline) are the most widely prescribed pharmacological treatment for depression. Since their introduction, many have considered the primary mechanism, by which the SSRIs produced therapeutic improvement in depression, is their effect on monoaminergic signaling.[1]

The prevalence of depression in elderly patients (those aged 65 years or older) may be as high as 40% in hospital and nursing home settings and 8-15% in community settings. Many studies show that depression and anxiety, if not treated adequately in older age groups, are associated with adverse outcomes, such as increased risk of disability, poor quality of life and mortality.[2-4] Because of the age related changes in pharmacokinetics and pharmacodynamics of drugs, it is not possible to automatically extrapolate findings on the efficiency or tolerability of antidepressants from younger to older populations. In the latter case, the risk of overdose and

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adverse effects should be considered, and a laboratory measurement of plasma SSRI levels becomes mandatory.[5,6]

Capillary electrophoresis (CE) is an established analytical method with a continuously growing potential for solving challenging problems, ranging from small ion to macromolecule analysis. The availability of various modes has made CE extremely useful for drug analysis, because pharmaceuticals are diverse in structures and physicochemical properties.[7] Non-aqueous capillary electrophoresis (NACE) is an interesting alternative to modify selectivity, the substitution of water by an organic solvent may have an impact on the charge of the analyte and/or its solvation size. The latter often governs selectivity modifications, as ion solvation is dependent on the solvent nature.[8]

Most of the NACE methods for analysis of antidepressants in biological fluids use laborious, time-consuming concentration techniques, such as solid-phase extraction (SPE) and liquid-liquid extraction (LLE), requiring large amounts of organic solvents.[9-12] Time- and labor-saving sample pretreatment methods that reduce the matrix content and enrich the sample with the target analytes are desirable. Such methods should also employ smaller amounts of solvents and samples, and should involve as few steps as possible in order to be more eco-friendly, minimize potential errors, and shorten the analysis time.[13] Some cleanup/concentration methods such as stir bar sorptive extraction (SBSE) and microextraction by packed sorbent (MEPS) are effective for preconcentration purposes and significantly enhance the detection sensitivity in CE.[13]

In SBSE, the extraction sorptive phase (generally, polydimethylsiloxane) is coated (0.5 to 1-mm layer) onto magnetic stir bars (1 to 4 cm in length) composed of a magnetic rod surrounded by a glass jacket. During stirring of the aqueous sample, analytes are extracted in accordance with their partition coefficients into the extraction phase. Although not fully correct, the octanol-water distribution coefficient gives a good indication if and how well a given solute can be extracted with SBSE. Desorption can be performed thermally or by liquid desorption in a few microliters of an organic solvent for back-extraction.[14]

MEPS consists of a 100 to 250 μL syringe containing 1 mg of packed sorbent (inserted into a barrel inside the syringe as a plug or between the barrel and needle as a cartridge). The sorbents are miniaturized to work with microliter bed volumes, enabling use of sample and elution volumes as low as 10 μL. The cartridge bed can be packed or coated to provide selective and suitable sampling conditions. Any sorbent material such silica based (C2, C8, C18) or strong cation exchanger (SCX) using sulfonic acid bonded silica can be used.[15]

The aim of this study was to critically evaluate the potential of SBSE and MEPS techniques to be used in the determination of the sertraline, fluoxetine, citalopram and paroxetine levels in plasma samples obtained from elderly depressed patient plasma samples using NACE analysis.

Experimental

Reagents and analytical standards

The fluoxetine and paroxetine analytical standards were kindly donated by Lilly (São Paulo, Brazil), and Libbs (São Paulo, Brazil), respectively. Roche provided citalopram, mirtazapine and sertraline standards (São Paulo, Brazil). The working standard solutions were prepared by diluting the stock solutions of these drugs (1 mg mL^{-1} in methanol) to an appropriate volume of methanol, based on their therapeutic intervals.

These solutions were stable for 45 days, at a temperature of –20 ºC. The water used to prepare the solutions had been previously purified in a Milli-Q system (Millipore, São Paulo, Brazil). Methanol, acetonitrile (all HPLC grade), and monobasic and dibasic phosphates were purchased from Merck (Darmstadt, Germany). Ammonium acetate, sodium chloride, sodium hydroxide and phosphoric acid were acquired from Mallinckrodt Baker (Xalostoc, Mexico).

Instrumentation and NACE procedure

NACE experiments were carried out in a Beckman P/ACE 5500 CE instrument (Beckman Coulter Inc., Fullerton, CA, USA) equipped with a diode-array (DAD) detector. Analytes were monitored at 200 nm with a bandwidth of 16 nm. NACE was performed in the normal mode, by applying a positive voltage, and using a fused silica capillary with 75 μm i.d. and total length of 40 cm. Samples were introduced hydrodynamically using 6.9 mbar (10 s). Before each injection, the capillary was conditioning with 1 mmol L^{-1} sodium hydroxide (2.0 min), water (2.0 min) and the separation electrolyte (2.0 min), respectively. NACE separation of drugs was carried out using phosphoric acid (1.25 mol L^{-1}) in acetonitrile as the background electrolyte (BGE).

Plasma samples

Drug-free plasma samples from patients that had not been exposed to any drug for at least 72 h (blank plasma) were kindly supplied by Hospital das Clínicas de Ribeirão Preto, University of São Paulo, Brazil. These plasma samples were spiked with the analytes and further

used to optimize the MEPS and SBSE processes, and in the validation of the analytical methods.

The plasma samples were collected from male geriatric patients (above 60 years old) that had been subjected to therapy with antidepressants for at least two weeks. Blood samples were withdrawn 12 h after the last drug administration. The principles embodied in the Helsinki Declaration were followed. These plasma samples were collected in agreement with the criteria established by the Ethics Committee of the University of São Paulo.

SBSE procedure

The commercial stir bar Twister® used for sorptive extraction was obtained from Gerstel (Gerstel GmbH, Mulheim an der Ruhr, Germany). The bar consists of a 10 mm length glass-encapsulated magnetic stir bar externally coated with 22 μg of polidimethylsiloxane (PDMS). This layer is 0.5 mm thick, which corresponds to a volume of 24 μL of PDMS. Prior to the first use, the stir bars were placed in a vial containing an acetonitrile:methanol solution (80:20, v/v), and conditioned for 24 h, under agitation. Between each extraction, the used stir bars were cleaned in methanol for 30 min at 50 °C, under magnetic stirring rate at 1200 rpm.

The effect of SBSE variables on extraction performance was evaluated on the basis of our previous work.[16] First, the influence of the sample pH on the extraction of antidepressants was assessed for pH values ranging from 7.0 to 11.0 (buffer solutions, 0.05 mol L^{-1}).

In a glass vial (5 mL), sealed with a silicone septum, 50 μL internal standard (10 μg mL^{-1}, mirtazapine) and 4.0 mL of the buffer solution were added to 800 μL of the plasma sample spiked with the standard solutions that resulted in a concentration of 500 ng mL^{-1}. The vial was heated up to 50 °C on a hotplate; the stir bar was immersed into the sample, and the extraction was carried out under magnetic stirring rate at 1200 rpm for 45 min. The influence of extraction time (15, 30, 45 and 60 min) and temperature (38, 50, 60 and 70 °C) on the SBSE performance was investigated simultaneously. For desorption, the stir bars were removed from the vials with clean tweezers, rinsed slightly with Milli-Q water, dried with lint-free tissue, and placed in a glass vial containing 1.0 mL of the acetonitrile, ensuring the total immersion at 50 °C for 15 min. After the desorption process, the stir bars were removed by means of a magnetic rod and the solvent (acetonitrile) was evaporated until dryness. This dry residue was redissolved in 50 μL of the acetonitrile, and an aliquot was injected in NACE-DAD system. After the desorption procedure, the carryover was evaluated by blank assay.

MEPS procedure

The MEPS procedure described here is based on our previous work.[17] The MEPS syringe (250 μL syringe, C8 and strong cationic exchange sorbent, 2 mg) was donated by SGE (Melbourne, Australia). This sorbent has irregular particles with average size of 50 μm and nominal of 60 Å porosity. Before being used for the first time, the sorbent had been manually conditioned with 250 μL methanol followed by 250 μL of water. After that, 400 μL of the plasma sample, diluted with 400 μL of the phosphate buffer solution 50 mmol L^{-1} (pH 4.0), were manually drawn through the MEPS sorbent and dispensed into the same vial three times (3 × 250 μL). The solid phase was then washed once with 250 μL of 0.1% formic acid in water to remove proteins and other interferences. The drugs were then desorbed (eluted) with 150 μL of phosphate buffer (50 mmol L^{-1}, pH 4.5) and methanol solution (55:45 v/v). Between extractions, the solid phase was washed 3 times with 250 μL of water followed by 250 μL of formic acid solution (0.01%) and of 250 μL methanol between sorbent extractions.

Analytical validation

The NACE methods were validated using blank plasma samples spiked with standard solutions at concentrations that included the therapeutic levels. The linearity of the methods was evaluated in concentrations ranging from limit of quantification (LOQ) values to 500 ng mL^{-1}. LOQ was considered the lowest concentration quantified with a coefficient of variation less than 10% that was obtained from five determinations. Calibration curves were generated for every analyte by plotting the relative peak areas (analyte-to-IS) as a function of the plasma sample concentrations.

Accuracy and inter-day precision were determined by quintuplicate microextraction/NACE assays of the blank plasma samples spiked with the analytes (quality control samples) that represented the entire range of the calibration curve. Accuracy values were calculated by comparison between the concentrations of the drugs added to the plasma samples with the plasma drug concentrations determined by the calibration curve.

Results and Discussion

NACE procedure

In general, NACE is selected when there is an inherent solubility or selectivity problems with the analytes. NACE, however, is seldom used for other purposes despite the

additional advantages that it bears, including the generation of reduced electric current, the possibility to perform rapid analyses, or even the efficient coupling with a variety of common detectors.[18] The optimization of the separation conditions for all analytes was investigated in an earlier investigation.[6] In brief, a variety of CZE, MEKC and even some NACE conditions were not able to satisfactorily achieve the separation of all four antidepressants along with the internal standard. Successful separation was only possible when the background electrolyte was plain phosphoric acid in acetonitrile.

Optimization of the SBSE variables

The major SBSE variables, such as time, temperature and pH of sample were optimized to establish the partition equilibrium conditions, and increase both the analytical sensitivity and the inter assay precision of the method. The sample volume, stirring speed and stir bar dimensions were maintained constant during the optimization.

The dilution of plasma samples with borate buffer (pH 9.0) favors the partition of the drugs (absorption) into the PDMS phase (Figure 1a). In agreement with the pKa values of the drugs (6.7-9.8), the extraction efficiency should be increased with sample pH values when increasing from 7.0 to 11.0. However, we observed a decrease on the extraction efficiency for plasma samples diluted with buffer solutions with pH above 9.0. Probably, at this condition, the sorption of the drugs into PDMS phase indeed increased, but the desorption procedure (1.0 mL of the acetonitrile at 50 °C for 15 min) was not efficient, leading to low recoveries.

The dilution procedure decreased the plasma sample viscosity and favored the drugs to diffuse into the bulk of the coating. Figure 1b shows a representative time extraction profiles (15-60 min) of fluoxetine at different temperatures (38-70 °C). The increase in the extraction temperature from 38 to 50 °C resulted in larger amounts of extracted drug, but further increments in the temperature (60-70 °C) decreased the extraction of the drugs. At lower temperature (38 °C), the extraction conditions are far from equilibrium, and at higher temperatures (60-70 °C), the partition coefficients of the drugs into PDMS phase decrease. As a result, the optimal equilibrium SBSE conditions were established at temperature of 50 °C for 45 min.

Carry-over and reuse of the microextraction devices

After SBSE and MEPS desorption procedures, no carryover was observed when followed by a blank assays (data not shown). Even though, a sorbent cleanup step was incorporated in the protocol procedures to increase the lifetime of the sorbent. Each sorbent was reused in plasma samples for multiple extractions, typically more than 50 times.

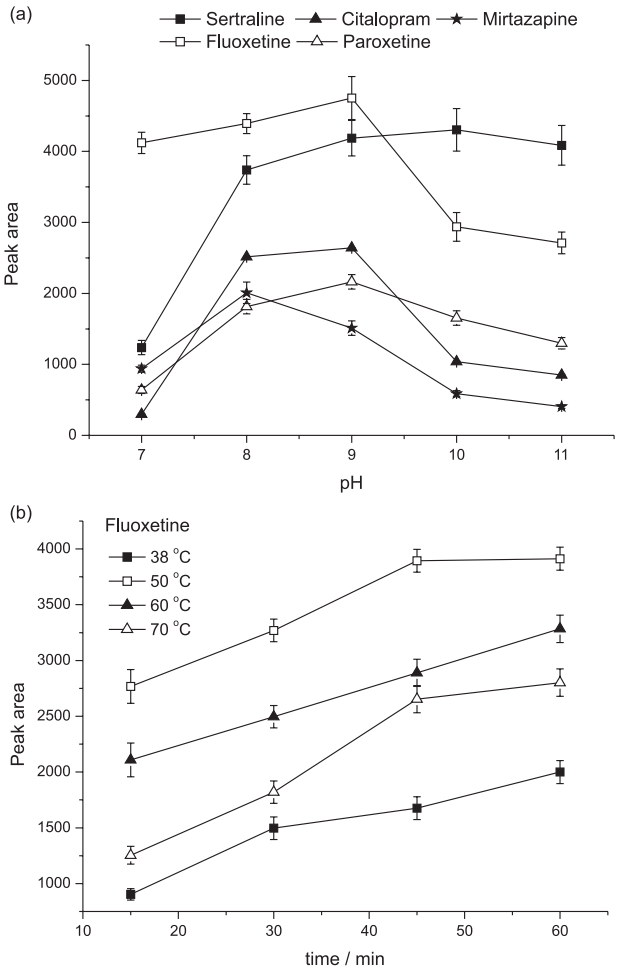

Figure 1. Optimization of the SBSE variables. (a) The effect of extraction pH on SBSE performance of all analytes, and (b) SBSE time extraction profiles for fluoxetine at different temperatures.

Analytical validation of NACE methods

Representative SBSE/NACE electropherograms of a blank plasma sample and the same sample spiked with antidepressants at therapeutic concentrations attested to the specificity of the developed methods (Figure 2).

The SBSE/NACE and MEPS/NACE electropherograms of the spiked plasma samples were similar and evidenced the ability of both methods to measure the drugs in the presence of plasma endogenous components. Blank plasma samples from several individuals were tested and it was not observed any significant interference at the migration times of the analytes.

The linearity of the SBESE/NACE and MEPS/NACE methods was determined with plasma samples spiked with

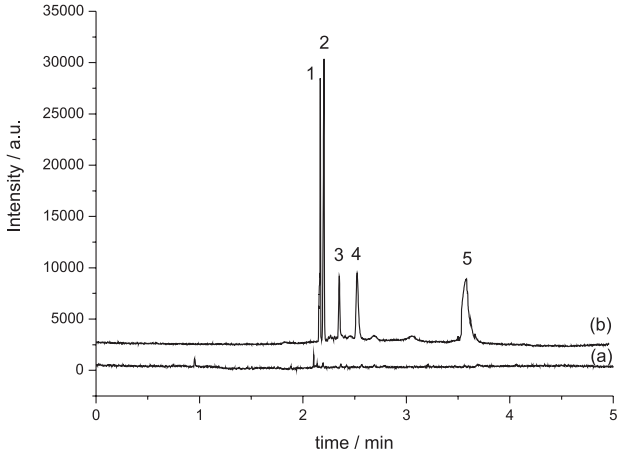

Figure 2. SBSE/NACE electropherograms obtained for (a) blank plasma sample and (b) blank plasma sample spiked with antidepressants at a concentration of 500 ng mL^{-1}. NACE conditions: BGE, 1.25 mol L^{-1} phosphoric acid in acetonitrile, hydrodynamic injection, 10 s, electric field, 500 V cm^{-1}, and temperature 25 °C. Peaks: (1) sertraline, (2) fluoxetine, (3) citalopram, (4) paroxetine and (5) mirtazapine (I.S.). The electropherograms were stacked and offset to facilitate visualization.

analytical standards that resulted in concentrations ranging from the LOQ for each antidepressant up to 500 ng mL^{-1}. Table 1 lists the regression equations and the corresponding correlation coefficients obtained for the drugs.

The LOQ values (Table 1) were determined as the lowest concentration of the calibration curves that can be measured with acceptable accuracy and precision (coefficient of variation was lower than 20%). Accuracy and inter-assay precisions of both methods were evaluated at three levels (high, medium and low) and the results are compiled in Table 2.

The results from the SBSE/NACE and MEPS/NACE methods presented here were compared with those from conventional liquid-liquid extraction method (LLE/NACE) from our earlier work,[6] Table 3.

The MEPS/NACE method presented shorter extraction time and lower CV values (inter-assay precision) than the other methods. LOQ values of the methods ranged from 10 to 30 ng mL^{-1}, and the reference LLE/NACE method gave slightly better accuracy values. However, all methods (LLE, SBSE and MEPS) presented accuracy values greater than 87%.

Compared to the reference method, LLE/NACE, the SBSE/NACE and MEPS/NACE methods consumed smaller volumes of sample (LLE: 1.0 mL, SBSE: 800 µL, and MEPS: 400 µL), used less organic solvents, and had fewer sample preparation steps, minimizing potential errors. Minimal volumes of organic solvents are used only on

Table 1. Linearity and LOQ values obtained for the SBESE/NACE and MEPS/NACE methods

Drug	SBSE/NACE method, linearity (LOQ - 500 ng mL^{-1})			LOQ / (ng mL^{-1})	MEPS/NACE method, linearity (LOQ - 500 ng mL^{-1})			LOQ / (ng mL^{-1})
	Intercept	Slope	Correlation (r)		Intercept	Slope	Correlation (r)	
Sertraline	0.181	7.100	0.998	10	0.0068	0.0202	0.998	20
Fluoxetine	0.177	8.073	0.997	20	0.004	0.0606	0.998	25
Citlopram	0.133	3.731	0.998	25	0.002	0.0486	0.999	30
Paroxetine	0.113	4.731	0.997	20	0.008	0.0311	0.997	25

Table 2. Inter-day precision (CV, coefficient of variation) and accuracy of the methods

Drug	Added concentration	SBSE/NACE method		MEPS/NACE method	
		Inter assay precision, CV / %, n = 5	Accuracy / % n = 5	Inter assay precision, CV / %, n = 5	Accuracy / % n = 5
Sertraline	50	10.5	95	8.5	97
	200	4.8	101	3.8	93
	500	3.2	99	3.2	95
Fluoxetine	50	12.6	98	7.6	95
	200	7.8	98	5.8	92
	500	2.9	97	3.9	94
Citalopram	50	11.6	96	8.7	97
	200	4.5	98	4.4	98
	500	4.3	97	4.0	99
Paroxetine	50	9.8	97	6.8	95
	200	7.6	96	4.6	97
	500	2.9	101	2.9	101

Table 3. Comparison of the SBSE/NACE, MEPS/NACE and LLE/NACE[6] methods with spectrophotometric detection

Drug	LOQ / (ng mL^{-1})			Accuracy / % (200 ng mL^{-1})			Inter assay precision, CV / % (200 ng mL^{-1}, n = 5)			Extraction time / min		
	LLE	SBSE	MEPS	LLE	SBSE	MEPS	LLE	SBSE	MEPS	LLE	SBSE	MEPS
Sertraline	20	10	20	97	95	93	4.7	4.8	3.8	45	45	3
Fluoxetine	15	20	25	96	88	92	5.9	7.8	5.8	45	45	3
Citalopram	30	25	30	97	87	98	6.2	4.5	4.4	45	45	3
Paroxetine	20	30	25	98	87	97	5.2	7.6	4.6	45	45	3

desorption procedures. Consequently, analyst exposure to biological fluids and toxic solvents also decreased.

The stir bar (SBSE) cleanup, step between each extraction, is longer (30 min) than the MEPS procedure (< 1 min). However, the manual MEPS procedure (draw/eject cycles) is one disadvantage of these technique. It is difficult in maintaining exactly the same conditions (i.e., sample flow rate through the sorbent).

Both MEPS (C8 + SCX phase) and SBSE (PDMS phase) are sorptive extractions, by nature, an equilibrium technique, that were governed by the partitioning coefficient of the drugs between the stationary phase and the biological sample. However, MEPS is a dynamic sampling, a diluted plasma sample was drawn (3 × 250 μL) through the sorbent (pre-concentration of the drugs) and eject into waste, and a SBSE requires mixing procedures such as stirring to promote diffusion of the drugs from the biological sample into PDMS phase.

Although static sampling (SBSE) can be an easy, reliable and straightforward technique, because it relies on the equilibrium distribution of compounds rather than on exhaustive extraction, care should be taken to ensure the distribution constant (K) is equal in all experiments, including calibration and sample extraction. Although this seems to be a simple requirement, in practice it is often not so. In chemical equilibria, temperature has a dominant effect on equilibrium and distribution constants, so careful control of the temperature; often within 1-2 °C is necessary. Conducting SBSE/NACE and MEPS/NACE methods under partition equilibrium conditions resulted in inter-assay precision with lower coefficient of variation.

Clinical application of the developed methods

In order to evaluate the proposed methods for clinical use, the described protocols were applied in the analysis of plasma samples from elderly depressed patients, (Figure 3). SBSE/NACE (not shown) and MEPS/NACE electropherograms obtained from plasma samples from patients under medication had no significant differences.

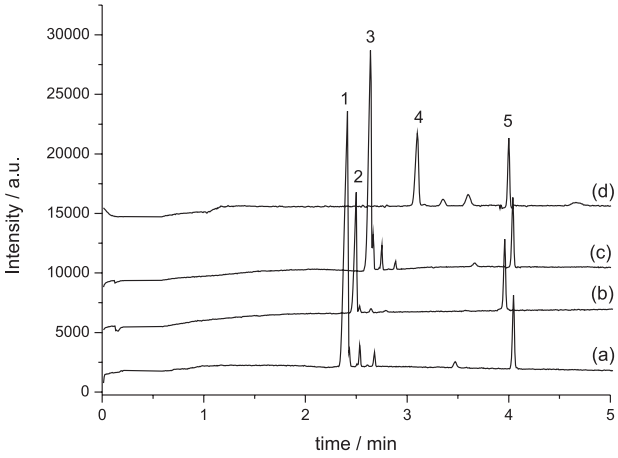

Figure 3. MEPS/NACE electropherograms obtained for elderly depressed patient plasma samples. Determined concentrations: (a): (1) sertraline (387.1 ng mL^{-1}); (b): (2) fluoxetine (220.1 ng mL^{-1}); (c): (3) citalopram (233.8 ng mL^{-1}); (d): (4) paroxetine (403.5 ng mL^{-1}); (5) mirtazapine (I.S.). NACE conditions: BGE, 1.25 mol L^{-1} phosphoric acid in acetonitrile, hydrodynamic injection, 10 s, electric field, 500 V cm^{-1}, and temperature 25 °C. Peaks: (1) sertraline, (2) fluoxetine, (3) citalopram, (4) paroxetine and (5) mirtazapine (I.S.). The electropherograms were stacked and offset to facilitate visualization.

Conclusion

The SBSE/NACE and MEPS/NACE methods presented high sensitivity, precision, accuracy and reduced matrix effect, allowing analysts to quantify antidepressants in human plasma following oral administration. MEPS/NACE method presented shorter extraction time (3 min), smaller volumes of plasma sample and lower CV values (inter-assay precision) than the other methods (LLE/NACE and SBSE/NACE). Thus, the proposed methods can be a useful tool to determine antidepressants in plasma samples obtained from patients receiving therapeutic dosages. The methods may also be applied in the evaluation of plasma levels in urgent toxicological analyses after the accidental or suicidal intake of higher doses.

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