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Simultaneous Determination of Antidepressants by Non-aqueous or Quasi-non-aqueous Capillary Electrophoresis

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Simultaneous determinations of 20 antidepressants were carried out by non-aqueous capillary electrophoresis using a background electrolyte consisting of an organic solvent. A bubble cell fused silica capillary (112.5 cm × 50 μm i.d., 150 μm i.d. bubble) was used as an electrophoresis tube. The determination was carried out at 215 nm, while the detection wavelength between 190 and 500 nm was selected for qualitative analysis. When an acetonitrile solution alone was used as the background electrolyte, good separation was observed, but it was not sufficient to separate all tested analytes. It was found that better separation was acquired by adding a few other solvents into acetonitrile, such as water and methanol; the best separation was achieved with a mixture of acetonitrile containing 60 mM ammonium acetate and 1 M acetic acid/water/methanol (100:1:0.5, v/v/v). As for the plasma sample, liquid–liquid extraction and solid-phase extraction (SPE) were considered; as a result, SPE with Oasis HLB was found to be most suitable. The present method is very useful as regards to plasma samples.

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Introduction

Patients with depression have been increasing steadily each year, and the consumption of antidepressants has also increased accordingly. Under this situation, a variety of antidepressants have been recently developed. Tricyclic antidepressants, such as amitryptyline, and tetracyclic antidepressants, such as maprotiline, belong to the classic psychotropic drugs, which are still frequently used together in psychiatric treatment. In recent years, selective serotonin reuptake inhibiters or serotonin noradrenalin reuptake inhibiters with fewer side effects are more frequently used.

From the viewpoint of forensic science, it was found that poisoning trouble occurred due to taking large amounts of an antidepressant; furthermore, incidents of suicide are many. Therefore, simultaneous determinations of these antidepressants are important in cases of taking an overdose antidepressant like this. Drug screening test kits are a widely used tool in the field of chemical trial, but biological samples are difficult to determine; it is impossible to distinguish some antidepressants. The simultaneous determination of antidepressants has so far been carried out by GC[1-6] and HPLC.[7-11] However, the simultaneous determination of all antidepressants by these methods is very difficult in a single run because of similar properties and structures.

Capillary electrophoresis (CE) is a separation method known for easy method development, low sample consumption, fast analysis times, simple experimental apparatus and high resolution. It is thus thought that CE is very effective for simultaneous determinations. However, because CE usually uses an aqueous migration buffer solution, it is not suitable for measuring compounds containing hydrophobic species, such as antidepressants, which must be devised. Besides, the determination of highly hydrophobic and structurally similar compounds by CE often requires the use of specific additives, such as a surfactant or an organic solvent in order to improve the separation selectivity.[12–18] Non-aqueous capillary electrophoresis (NACE) has emerged as an alternative to obtain the high-resolution separations of hydrophobic drugs.[19-22] The NACE method uses an organic solvent instead of an aqueous buffer as the background electrolyte, which is useful for antidepressants. However, there are very few reports on the determination of antidepressants in a single run by CE in recent years.[23-26]

Therefore, this work aims at the development of a simultaneous determination of 20 antidepressants, as shown in Fig. 1, using NACE. The compositions of the background electrolyte and other experimental conditions were optimized. Subsequently, the preparation for a plasma sample was investigated, and the benefit of this technique was suggested.

Experimental

Chemical

Amitriptyline (AMI), trimipramine (TRI), imipramine (IMI), desipramine (DES), clomipramine (CLO), nortriptyline (NOR), amoxapine (AMO), maprotiline (MAP), mianserin (MIA), fluvoxamine (FLU), milnacipran (MIL), trazodone (TRA) and sulpiride (SUL) were purchased from Sigma-Aldrich (St. Louis, MO). Paroxetine (PAR), sertraline (SER), flurazepam, methanol (HPLC grade), acetonitrile (HPLC grade), acetic acid (HPLC

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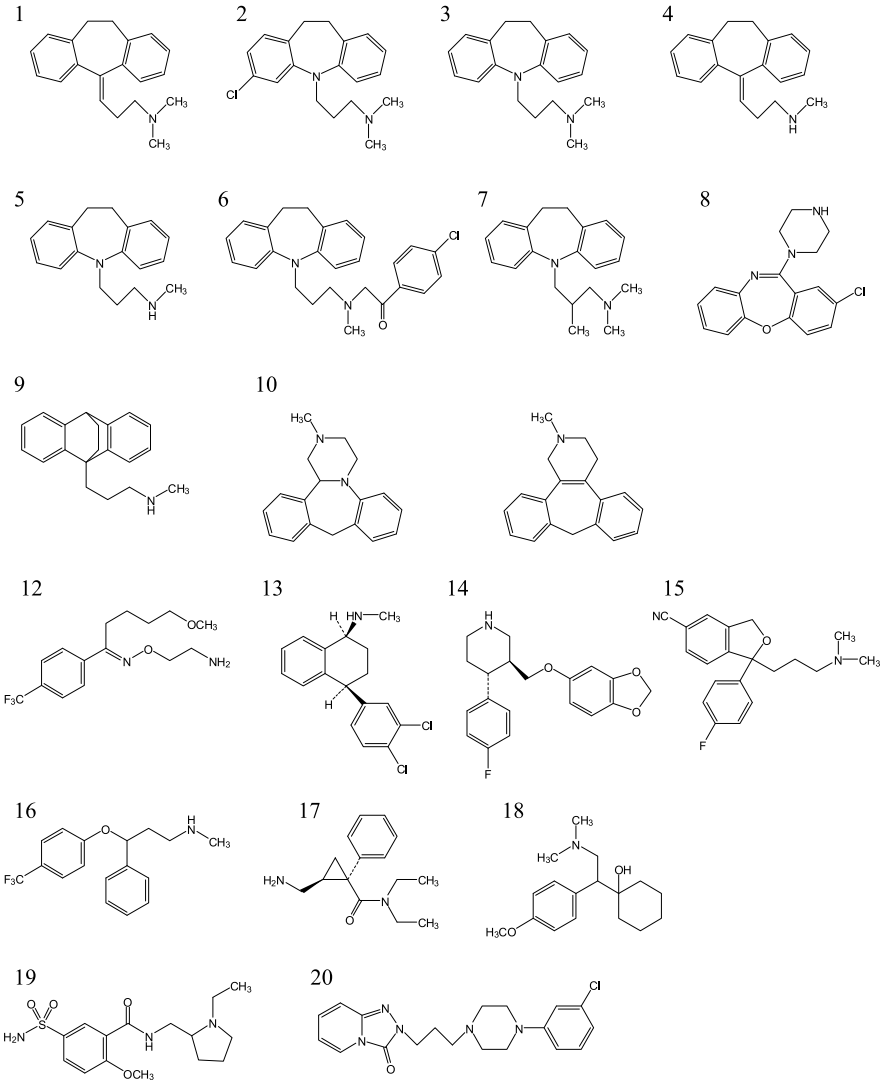

Fig. 1 Structures of the 20 antidepressants. (1) Amitriptyline (AMI), (2) clomipramine (CLO), (3) imipramine (IMI), (4) nortriptyline (NOR), (5) desipramine (DES), (6) lofepramine (LOF), (7) trimipramine (TRI), (8) amoxapine (AMO), (9) maprotiline (MAP), (10) mianserin (MIA), (11) setiptiline (SET), (12) fluvoxamine (FLU), (13) sertraline (SER), (14) paroxetine (PAR), (15) citalopram (CIT), (16) fluoxetine (FLO), (17) milnacipran (MIL), (18) venlafaxine (VEN), (19) sulpiride (SUL), (20) trazodone (TRA).

grade), and ammonium acetate (analytical grade) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Citalopram (CIT), fluoxetine (FLO) and venlafaxine (VEN) were purchased from LKT Laboratories Inc. (St. Paul, MN). Lofepramine (LOF) and setiptiline (SET) were obtained free-of-charge from Daiichi-Sankyo Co., Ltd. (Tokyo, Japan) and Mochida Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively. Oasis HLB, Oasis MCX and Oasis WCX extraction cartridges were purchased from Waters (Milford, MA). Bond Elute Plexa and Bond Elute Certify extraction cartridges were purchased from Varian (Palo Alto, CA). All other common chemicals were commercially available and of reagent grade.

Instrumentation and CE procedures

CE experiments were carried out in an HP 3DCE instrument (Agilent Technologies, Waldbron, Germany) equipped with a diode-array detector. Analytes were detected from 190 to 500 nm, and determination was carried out at 215 nm with a bandwidth of 10 nm. CE was performed in a normal mode, by applying a 30-kV positive voltage, using a bubble cell fused silica capillary (50 μm i.d.; bubble cell i.d., 150-μm i.d.) with a total length of 112.5 cm (104 cm effective length). The temperature of the capillary was controlled at 25°C. Samples were introduced hydrodynamically using 50 mbar. The capillary was washed with 0.1 M NaOH and distilled water for 10 min when it was not used for a long time. Before every injection, column conditioning was performed with acetonitrile (5.0 min) as well as a running buffer (5.0 min).

Sample preparation

The 20 antidepressants were individually stocked in acetonitrile at –4°C, where they were stable for 6 months. However, when these standard solutions were mixed up, *e.g.* 200 μg/ml of each compound in acetonitrile, LOF was resolved after a few days. When this standard solution (20 antidepressants) was stored at –20°C, it was stable for one month. Human plasma samples were spiked with the standard solution.

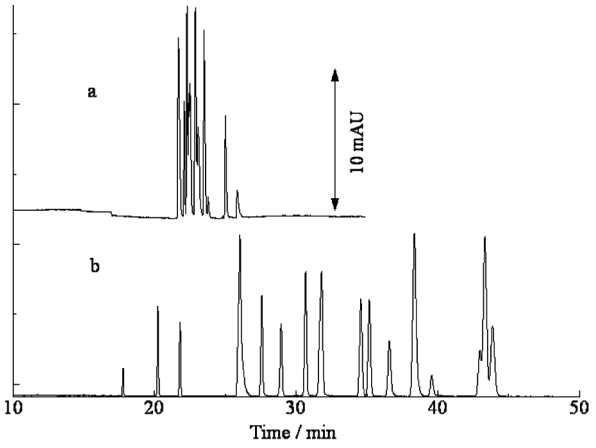

Fig. 2 Comparison of the aqueous CE method and the non-aqueous CE method. Capillary, fused-silica capillary, 50 µm i.d., 112.5 cm of the total length, 104 cm of the effective length. Applied voltage, 30 kV. Sample injection, hydrodynamic injection, 15 s at 50 mbar. Wavelength of detection, 215 nm. Sample, 10 µg/ml of each prepared in acetonitrile. Background electrolyte solution: (a) 0.2 M acetate aqueous buffer, pH 4.4, (b) 1 M acetic acid and 50 mM ammonium acetate in acetonitrile.

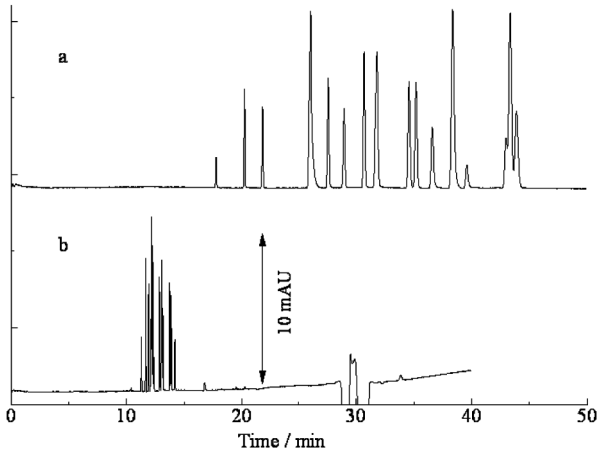

Fig. 3 Effect of difference acid and electrolyte. Other conditions are the same as in Fig. 2. Background electrolyte solution: (a) 1 M acetic acid and 50 mM ammonium acetate in acetonitrile, (b) 1 M formic acid and 50 mM ammonium formate in acetonitrile.

Results and Discussion

NACE method optimization

NACE and aqueous CE. The results of antidepressants measured by NACE (1 M acetic acid and 50 mM ammonium acetate in acetonitrile) and aqueous CE methods (acetate buffer, pH 4.4) are shown in Fig. 2. Both of the methods did not perform sufficient separation of all compounds. However, it was found that the NACE method was better in resolution and sensitivity than the aqueous CE method, and is more favorable for hydrophobic analytes, like antidepressants. It should be noted that a lower noise level is achieved for NACE, compared with aqueous CE.

Effect of electrolyte. The effect of an electrolyte was investigated. Differences in the concentration and the nature of electrolyte resulted in varied migration times and selectivity in NACE, and likewise in aqueous CE. For example, Fig. 3 demonstrates electropherograms obtained by using different electrolytes. A great difference in the migration time was observed between ammonium acetate and ammonium formate in acetonitrile. This difference in the migration time may be caused by a variation of the EOF magnitude because the background electrolyte changed the conductivity and pH* value (apparent pH), *e.g.*, 1 M formic acid and 50 mM ammonium formate in acetonitrile, pH* 2.83; 1 M acetic acid and 50 mM ammonium acetate in acetonitrile, pH* 4.81.[25,27] Most salts can hardly dissolve in acetonitrile directly. When citrate and phosphate aqueous buffer were mixed with acetonitrile, low resolution was observed when the composition of aqueous buffer was higher. The higher is the concentration of the electrolyte, the longer is the analysis time and the better is the resolution. However, the solubility of the electrolyte is poor in acetonitrile. In the case of acetonitrile, acetonitrile containing 1 M acetic acid and 50 or 60 mM ammonium acetate was most suitable for the NACE buffer.

Change of non-aqueous solvent. Non-aqueous solvents mixtures are widely used in NACE. The selectivity of the separation systems changed significantly with the ratio of non-aqueous solvents, and the electrophoretic mobility varied according to the composition of non-aqueous solvents. The characteristics of the non-aqueous solvent were investigated for methanol, acetonitrile and isopropanol. These solvents are suitable for direct UV detection (quantitative wavelength was 215 nm) because they have a little UV absorption. However, when a higher rate of isopropanol was used, the current could not flow, and a measurement was impossible. The results using several acetonitrile/methanol mixtures containing 50 mM ammonium acetate and 1 M acetic acid are shown in Fig. 4. Any conditions achieved symmetric and narrow peaks, although the migration time and selectivity were different. These results were caused by differences in the viscosity and the dielectric constant from that of non-aqueous solvents as well as by the difference in the dissociation constant of analyte molecules in non-aqueous solvents. The EOF velocity (v) can be estimated from

$$v = (\varepsilon\zeta/\eta)E,$$

where ε is the dielectric constant, ζ the zeta potential, η the solution viscosity, and E the applied electric field. For example, the viscosity values of acetonitrile–methanol mixtures at 25°C are 0.340, 0.329, 0.339 and 0.542 cP for 0, 22, 68 and 100% methanol, respectively, whereas the dielectric constants of acetonitrile–methanol mixtures at 25°C are 35.95, 35.38, 34.98 and 32.62 for 0, 27, 44 and 100% methanol, respectively.[28] The shortest migration time was observed with acetonitrile–methanol (75:25), but the resolution was poor. Since pure acetonitrile had the best resolution among the examined solvents, it was chosen in our study.

Effect of additive solvents. So far, it was observed that acetonitrile containing acetic acid and ammonium acetate achieved good resolution. However, the separations of some peaks were still insufficient. Unexpectedly, it was found that the migration behavior was changed when some amounts of water or organic solvent were added to the background electrolyte. The effect of additional solvents on the migration pattern is shown in Fig. 5. The addition of 1% water in acetonitrile showed a better resolution in comparison with no addition. However, a few peaks could not be separated when added water was more than 2%. Furthermore, a greater improvement in the separation was observed when adding 1% water and 0.5 to 1.0% methanol. It was thought that this

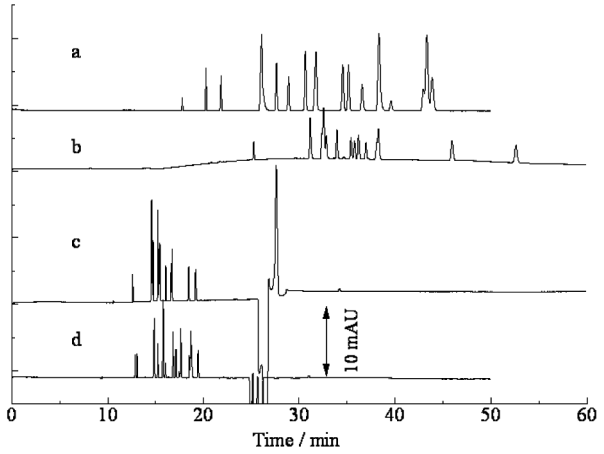

Fig. 4 Comparison with the difference mixture rate of acetonitrile and methanol. Other conditions are the same as in Fig. 2. Background electrolyte, 1 M acetic acid and 50 mM ammonium acetate. Medium: (a) 100% acetonitrile, (b) 100% methanol, (c) 75% acetonitrile and 25% methanol, (d) 50% acetonitrile and 50% methanol.

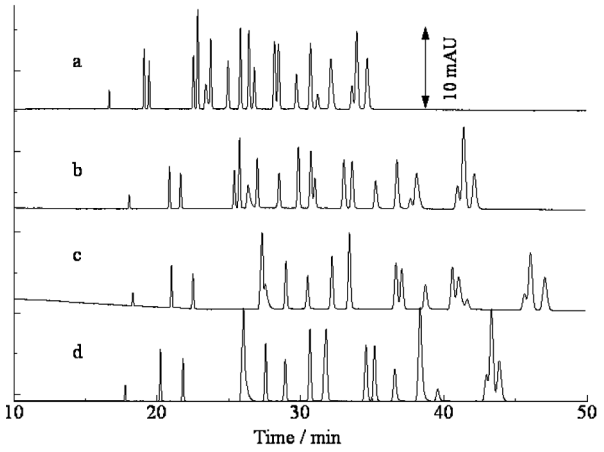

Fig. 5 Addition of a small amount of solvent to non aqueous background electrolyte. Other conditions are the same as in Fig. 2. Base of background electrolyte solution, 1 M acetic acid and 60 mM ammonium acetate in acetonitrile. Additional solvent: (a) 0.5% methanol and 1% water, (b) 1% water, (c) 1% methanol, (d) no addition.

tendency of separation was caused by a difference in the dissociation equilibrium of the analyte due to solvation with the additional solvents.

Applied voltage and capillary temperature. The effect of the applied voltage was investigated from 15 to 30 kV. The larger was the applied voltage, the higher was the achieved resolution. In addition, the current remained low even at high voltage in NACE, *e.g*. the current was about 10 μA at 30 kV. Therefore, the applied voltage was kept at 30 kV in the following experiments.

The effect of temperature on the separation was investigated in the range of 25 – 40°C because the capillary temperature could not be reduced to lower than room temperature. The analysis times decreased at higher temperature, resulting in poor separation. The capillary was set at room temperature (25°C).

Dissolving solution and injection volume. When the analytes

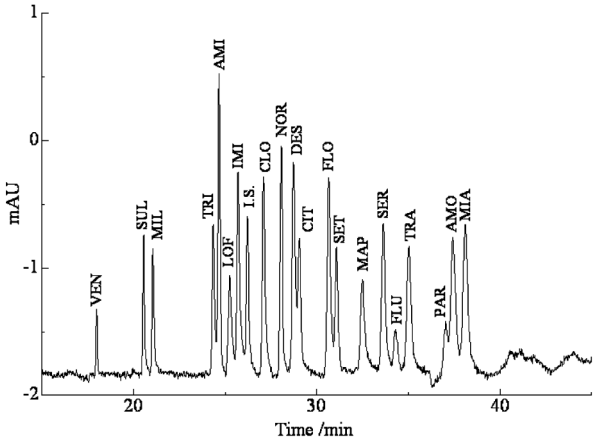

Fig. 6 Typical separation of plasma sample at the optimized conditions. Other conditions are the same as in Fig. 2. Sample, preparation for 0.1 μg/ml of plasma sample by solid phase extraction (Oasis HLB). Background electrolyte solution, 1 M acetic acid and 60 mM ammonium acetate in acetonitrile:water:methanol (100:1:0.5, v/v/v). Internal standard (I.S.), flurazepam.

were dissolved in the background electrolyte, a remarkably worse peak shape and resolution were observed for large injection volumes. Acetonitrile, methanol and chloroform were examined as the solvent-dissolving analytes. Since the sample was stacked at an interface between a low-conductivity sample zone and a high-conductivity background electrolyte, the peak shape was improved. The migration time was different for each solvent due to variations in EOF. One explanation for this may be that the electrical potential gradient was changed by the solvent conductivity. It is expected that a higher potential gradient is generated owing to low-conductivity sample solvents, which leads to the generation of higher EOF as a whole. The peak area was almost the same. This is because when the background electrolyte was the same, the injection volume was the same without regard to dissolving the solvent. The volume in the hydrodynamic mode was examined in the range of 5 – 30 s at 50 mbar. The migration time became short when a long injection time caused a changing potential gradient. The peak width had little deterioration up to 30 s injection at 50 mbar. Considering the migration time and the separation efficiency, the best separation was achieved with 15-s injection at 50 mbar. Under these optimized conditions, the simultaneous separation of 20 antidepressants was demonstrated (Fig. 6).

Bubble cell fused silica capillary. CE has a defect of low detection sensitivity caused by the very short detection pathlength. In order to extend the light path, a bubble cell capillary was tested. It should be noted that increasing the inner diameter of capillary leads to an increase in the current, and subsequent heating inside the capillary. The bubble cell is made by expanding the part of the capillary. The bubble cell extended the pathlength significantly without degrading the separation efficiency and the resolution. As a result, using a fused-silica capillary with an inner diameter of 50 μm and a bubble of 150 μm i.d., the peak area was on average 2.3-times higher compared with an unmodified capillary. The sensitivity was simply improved, with almost no variation in the separation efficiency. For example, the peak widths of VEN were 3.7 and 3.1 s for the straight cell and the bubble cell, respectively, while the peak widths of SET were 6.4 s for both cells.

Optimization of internal standard. It is difficult for us to control

the EOF in both aqueous CE and NACE. An internal standard was used for checking for any error of the migration time. For this purpose, flurazepam was selected as the internal standard because it could be detected without overlapping with the other analyte peaks. The relative standard deviations of the migration time were all less than 0.5% when flurazepam was used as the internal standard.

The identification of each antidepressant was achieved by comparing the revised migration time and the absorbance spectrum from 190 to 500 nm.

Table 1 Recovery of extraction by Oasis HLB, limits of detection (LOD), limits of quantification (LOQ), treatment and toxic plasma concentrations[29] (n = 5)

Analyte	Rec., %	R.S.D., %	LOD/ μg ml^{-1}	LOQ/ μg ml^{-1}	Treatment level/ μg ml^{-1}	Toxic level/ μg ml^{-1}
VEN	93.9	4.9	0.03	0.1	0.25 – 0.75	>1
SUL	70.5	5.9	0.01	0.03	0.40 – 0.60	38
MIL	90.4	6.3	0.01	0.03	0.15	
TRI	92.8	3.1	0.01	0.03	0.015 – 0.051	>1
AMI	92.7	3.2	0.01	0.03	0.05 – 0.24	0.55 – 16.1
LOF	54.3	11.7	0.03	0.1	0.04 – 0.14	
IMI	85.3	2.2	0.01	0.03	0.01 – 0.08	0.8 – 13
CLO	91.2	2.5	0.01	0.03	0.02 – 0.07	>0.4
NOR	86.1	2.2	0.01	0.03	0.05 – 0.15	>0.25
DES	101.3	3.1	0.01	0.03	0.02 – 0.88	>10
CIT	88.7	3.2	0.01	0.03	0.02 – 0.2	0.5
SET	93.1	3.0	0.01	0.03	0.0035 –	0.78 – 1.8
MAP	87.8	3.9	0.01	0.03	0.05 – 0.24	6.2
FLO	97.3	3.7	0.01	0.03	0.15 – 0.5	1.3 – 6.8
SER	90.8	4.7	0.01	0.03	0.05 – 0.25	2.9
FLU	93.2	14.7	0.01	0.1	0.05 – 0.25	0.16 – 1.4
TRA	98.6	8.0	0.01	0.03	0.49 – 2.3	15
PAR	100.3	5.4	0.03	0.1	0.01 – 0.075	>0.4
AMO	64.6	8.6	0.01	0.03	0.02 – 0.09	0.26 – 6.7
MIA	86.9	5.1	0.01	0.03	0.015 – 0.07	>0.5

Plasma sample

Solid phase extraction and liquid–liquid extraction. Solid phase extraction (SPE) and liquid–liquid extraction (LLE) were investigated for preconditioning the plasma sample. LLE was studied for ethyl acetate, chloroform–isopropanol (3:1, v/v), diethyl ether and *n*-hexane. As for neutral plasma samples, several analytes had low recovery, especially for MIL and SUL. When extracting by diethyl ether at pH >12 (addition of sodium carbonate or ammonia solution), the recovery was the best. However, the recovery of MIL and SUL were still low, *i.e.* about 40 and 20%, respectively, whereas the other analytes had more than 80% recoveries. Nevertheless, no interrupting peak derived from biological samples was detected.

SPE was studied using Oasis HLB, Oasis MCX, Oasis WCX, Bond Elute Plexa and Bond Elute Certify. Oasis HLB seems to have had the best recovery among the examined materials; the results are summarized in Table 1. In the case of the mixed-mode SPE (Oasis MCX, Oasis WCX and Bond Elute Certify), some analytes were eluted during washing, and some even strongly held and were not eluted at all. Bond Elute Plexa was able to recover all analytes, but the recovery was low overall. SPE using Oasis HLB was chosen in this study, and the procedures were as the follows: the cartridges were activated with methanol (1 ml), followed by water (1 ml). It was then loaded with 1 ml of human plasma containing a spiked standard solution and the internal standard (flurazepam, 10 μg/ml, 10 μl) with the addition of 20 μl phosphoric acid. The cartridge was then washed with 5% methanol (1 ml). The elution solvent was methanol. It was dried under a stream of nitrogen gas while heating at 40°C. The residue was dissolved in acetonitrile for assay.

Linearity, reproducibility, limits of detection (LODs), and limits of quantification (LOQs). The linearity of the response was examined by the injection of spiked plasma samples after an SPE treatment. The linearity was tested over the range from

Table 2 Linearity parameters and the reproducibility (0.1 μg/ml plasma samples)

Analyte	Linearity range/ μg ml^{-1}	Intercept	Slope	Coefficience of correlation	Intraday R.S.D., % (n = 6)		Interday R.S.D., % (n = 6)	
					Migration time	Area	Migration time	Area
VEN	0.1 – 1	0.0050	2.3265	0.9987	0.30	4.3	0.75	4.7
SUL	0.03 – 1	0.1141	4.9290	0.9915	0.19	16.4	0.38	16.2
MIL	0.03 – 1	0.0732	5.5966	0.9981	0.17	4.6	0.35	4.6
TRI	0.03 – 1	0.0530	8.0923	0.9994	0.06	1.2	0.18	3.9
AMI	0.03 – 1	0.1330	14.5557	0.9994	0.05	1.0	0.13	4.7
LOF	0.1 – 1	–0.7892	9.4321	0.9902	0.07	13.8	0.31	9.2
IMI	0.03 – 1	0.1529	12.6300	0.9992	0.01	0.7	0.06	4.6
CLO	0.03 – 1	0.1171	12.8137	0.9994	0.03	1.2	0.06	3.0
NOR	0.03 – 1	0.1359	13.4977	0.9991	0.06	1.8	0.22	4.9
DES	0.03 – 1	0.4458	13.6363	0.9991	0.08	1.9	0.25	4.6
CIT	0.03 – 1	0.2248	8.1778	0.9997	0.09	1.6	0.24	2.4
SET	0.03 – 1	0.1493	14.9495	0.9993	0.14	1.0	0.23	1.3
MAP	0.03 – 1	0.1481	8.9356	0.9989	0.15	1.2	0.39	5.4
FLO	0.03 – 1	0.0293	8.4473	0.9985	0.18	5.7	0.48	11.9
SER	0.03 – 1	0.1246	11.7936	0.9985	0.21	2.6	0.47	7.8
FLU	0.1 – 1	0.0762	3.2823	0.9904	0.23	5.0	0.58	12.4
TRA	0.03 – 1	0.2015	12.3242	0.9965	0.23	9.4	0.40	6.7
PAR	0.1 – 1	0.1233	4.1426	0.9974	0.29	10.8	0.77	11.6
AMO	0.03 – 1	0.5958	12.8207	0.9955	0.31	9.1	0.79	8.3
MIA	0.03 – 1	0.2348	14.9067	0.9994	0.32	2.3	0.60	4.0

LOQ (0.1 or 0.03) to 1 μg/ml for each analyte in the plasma (1 ml of plasma samples). The results were given in terms of the relative peak areas. The linear-regression equations obtained using the least-squares method and the coefficients of the correlation are presented in Table 2.

The reproducibility was evaluated by intraday and interday assay regarding the relative migration times and the relative peak areas. The reproducibility using a 0.1 μg/ml plasma sample is shown in Table 1. As shown in Table 1, the migration time reproducibility is sufficient for all analytes. From the migration time together with the spectrum from 190 to 500 nm, it was possible to identify the analyte.

LODs and LOQs were calculated by measuring the ratio of the signal to noise, and taking into account a factor of 3 and 10 for LODs and LOQs, respectively (Table 1); 0.01 to 0.03 and 0.03 to 0.1 μg/ml were achieved for LOD and LOQ, respectively. The concentration of the treatment level and the toxic level for antidepressants are also summarized in Table 1. At the concentration of the toxic level, all of the antidepressants could be determined. At the concentration of the treatment level, all of the antidepressants, except for SET, could be detected.

Conclusion

It was possible to simultaneously analyze 20 antidepressants using the developed NACE method, and this method was successfully applied to plasma samples. It was found that this NACE method could be sufficiently adapted to plasma samples.

The NACE method has a benefit for hydrophobic compounds. Since the consumption of the organic solvent and the sample is very little, this proposed NACE method is recommended as the first choice for hydrophobic drug screening. Coupling of the present NACE method with mass spectrometry is being investigated.

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