II.3.3 Tricyclic and tetracyclic antidepressants

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Introduction

Many of antidepressants exert their effects by inhibiting the reuptake of norepinephrine and serotonin and by accerelating the release of them at synaptic terminals of neurons in the brain. As characteristic structures of such drugs showing antidepressive effects, many of them have tricyclic or tetracyclic nuclei; this is the reason why they are called "tricyclic antidepressants or tetracyclic antidepressants".

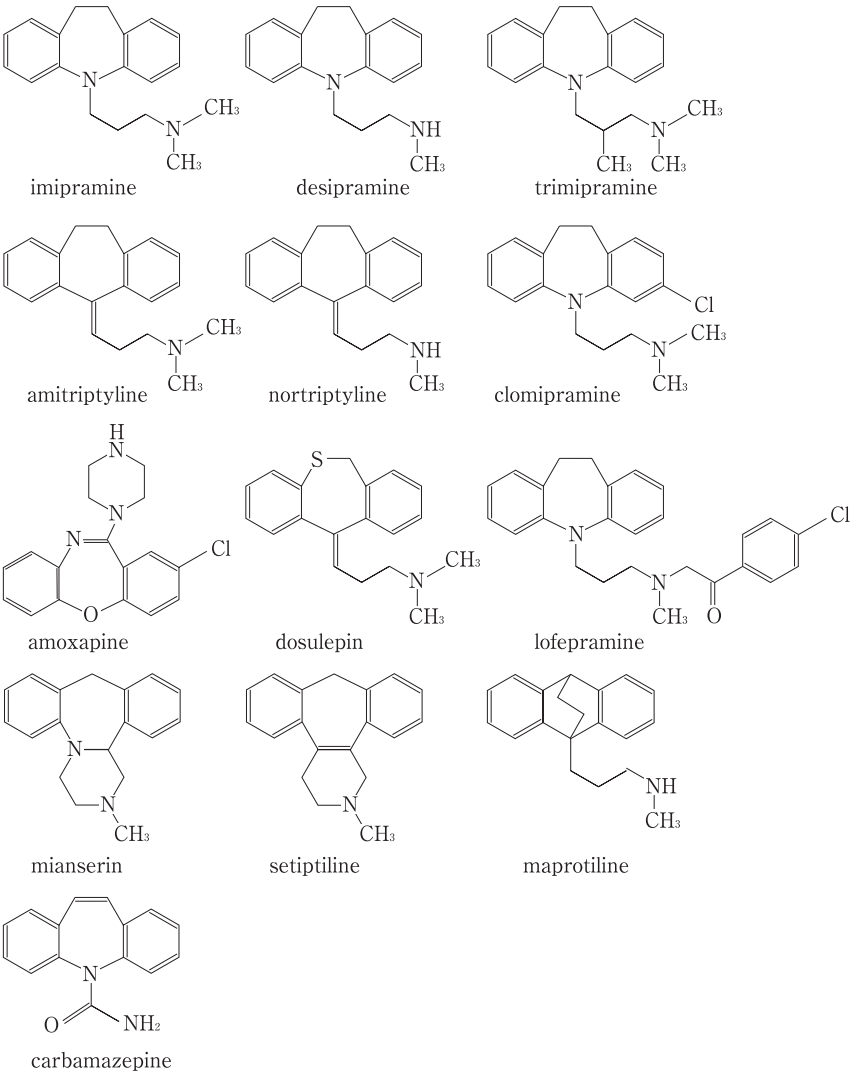
There are many cases of suicides using the antidepressants; their massive intake sometimes causes death. About 10 kinds of tricyclic and tetracyclic antidepressants are now being used in Japan (*Figure 3.1)*; among them, amitriptyline is best distributed [1, 2]. Recently, the use of tetracyclic antidepressants is increasing, because of their mild side effects and their high effectiveness with their small doses; the increase of their use is causing the increase of their poisoning cases. Although carbamazepine does not belong to the antidepressant group, its structure is very similar to those of tricyclic antidepressants; therefore, the drug is also included in this chapter.

GC/MS analysis

Reagents and their preparation

- Amitriptyline, carbamazepine, clomipramine, desipramine, imipramine, maprotiline, mianserin, nortriptyline and trimipramine can be purchased from Sigma (St. Louis, MO, USA); pure powder of the following drugs was donated by each manufacturer: amoxapine by Takeda Chem. Ind. Ltd., Osaka, Japan; dosulepin by Kaken Pharmaceutical Co., Ltd., Tokyo, Japan; lofepramine by Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan; and setiptiline by Mochida Pharmaceutical Co., Ltd., Tokyo, Japan.
- A 20-g aliquot of sodium carbonate is dissolved in distilled water to prepare 100 mL solution (20 %, w/v).
- A 9.85-mL volume of hexane is mixed well with 0.15 mL isoamyl alcohol to prepare an extraction solvent.
- A 1-mg aliquot of promethazine (Sigma) is dissolved in 10 mL acetonitrile to prepare internal standard solution (0.1 mg/mL).

Figure 3.1



Structures of tricyclic and tetracyclic antidepressants and carbamazepine.

GC conditions

GC column: an HP-5MS fused silica capillary column (30 m × 0.25 mm i. d., film thickness 0.25 μm, Agilent Technologies, Palo Alto, CA, USA).

GC/MS conditions; instrument: an HP 5890 Series II gas chromatograph (Agilent Technologies) connected with a mass spectrometer (HP-5971A MSD, Agilent Technologies); column (oven) temperature: 170 °C (1 min) → 5 °C/min → 280 °C (4 min); injection temperature: 250 °C; detection temperature: 280 °C; carrier gas: He (100 kPa); mass scan range: *m/z* 50–500.

Procedure

i. A 0.5-g (mL) aliquot of a specimen[a], 0.5 mL dissolved water, 0.2 mL of 20 % sodium carbonate solution and 25 μL of promethazine solution (0.1 mg/mL, IS[b]) are placed in a 10-mL volume glass centrifuge tube with a ground-in stopper and mixed well[c].
ii. A 3-mL volume of hexane/isoamyl alcohol[d] (98.5:1.5, v/v) is added to the mixture and shaken vigorously for 2 min.
iii. The tube is centrifuged at 3,000 rpm for 3 min.
iv. A 2.5-mL volume of the upper organic phase is transferred to a 8-mL volume glass vial and evaporated to dryness under a stream of nitrogen.
v. The residue is dissolved in 0.5 mL hexane and a 1-μL aliquot of it is injected into GC/MS.

Assessment of the method

Figure 3.2 shows total ion chromatograms (TICs) obtained by GC/MS for tricyclic and tetracyclic antidepressants (5 μg/mL) spiked into human whole blood. Using the slightly polar capillary column (HP-5MS), the peak top of trimipramine could be separated from that of imipramine, but they were not separated at the bottom completely. With non-polar columns, many drugs could not be separated from each other; such a type of columns seems not suitable for analysis of a specimen, which may contain multiple antidepressant drugs. The intermediately polar columns may be useful for drugs, which are not separable with non-polar or slightly polar columns, but in these experiments, only the conditions using a slightly polar capillary column are presented, because of its wide applicability to various drugs. Using the present TIC, the detection limit was about 0.01 μg/g (mL); this means that toxic and fatal levels of the drugs can be detected by this method.

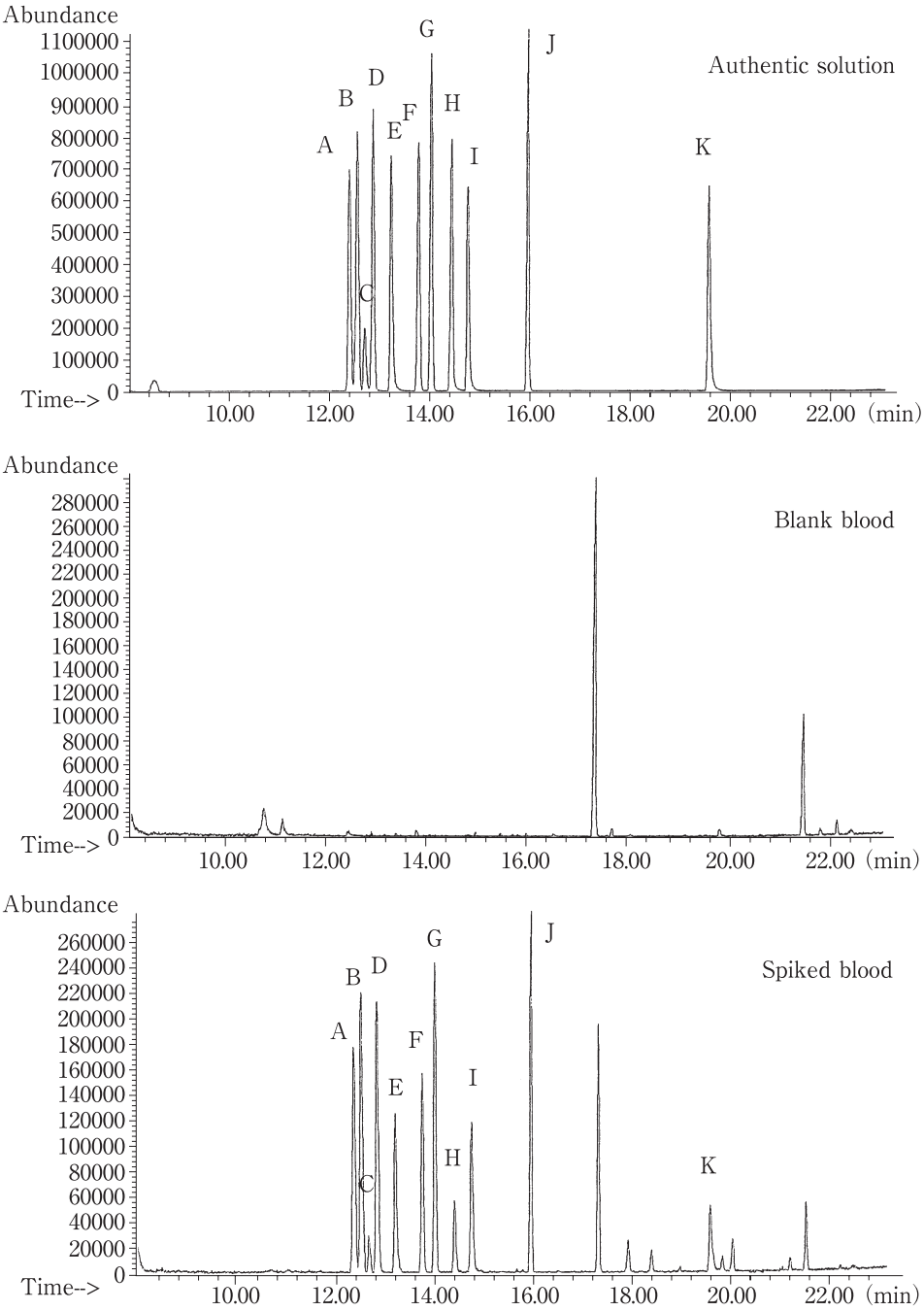
Many of tricyclic antidepressants are demethylated and/or hydroxylated to be converted into active metabolite(s)[e]. Therefore, to assess a blood concentration of an antidepressant, the combined concentration of the drug itself plus active metabolite(s) should be considered.

HPLC analysis

Reagents and their preparation

- Phosphoric acid used is of the special grade commercially available.
- An Oasis HLB[f] column (30 mg/cc, Waters, Milford, MA, USA) is activated by passing 1 mL methanol and 1 mL distilled water.
- A 0.5-mL volume of methanol is mixed with 9.5 mL distilled water to prepare 5 % methanol solution (v/v).
- A 2-g aliquot of phosphoric acid is dissolved in 1,000 mL of Milli Q water[g]; the pH of the solution is adjusted to 3.0 by adding NaOH aqueous solution.
- A 1.22-g aliquot of sodium dihydrogenphosphate (dihydrate, $NaH_2PO_4 \cdot 2H_2O$) and 1.73 g disodium hydrogenphosphate are dissolved in 1,000 mL of Milli Q water; the pH of the

Figure 3.2



TICs for tricyclic and tetracyclic antidepressants and carbamazepine obtained by GC/MS. A: amitriptyline, B: mianserin, C: nortriptyline, D: imipramine, E: desipramine, F: promethazine (IS), G: setiptiline, H: carbamazepine, I: maprotiline, J: clomipramine, K: amoxapine.

solution is adjusted to 6.5 by adding either NaOH aqueous solution or phosphoric acid to prepare 20 mM phosphate buffer solution (pH 6.5).

- Mobile phase (A): 50 mL acetonitrile is well mixed with 450 mL of 0.2 % phosphoric acid. After degassing[h], the solution is passed through a filter (0.45 μm) to be used as a mobile phase.
- Mobile phase (B): 300 mL acetonitrile is well mixed with 200 mL of 20 mM phosphate buffer solution (pH 6.5). After degassing, the solution is passed through a filter (0.45 μm) to be used as a mobile phase.

HPLC conditions

HPLC column[i]: an Eclipse XDB-C_8 octyl group bonded silica column (250 × 4.6 mm i. d., particle size 5 μm, Agilent Technologies).

HPLC conditions: an LC-10A high-performance liquid chromatograph (Simadzu Corp., Kyoto, Japan); detectors: a UV-VIS detector (UV-VIS, Shimadzu Corp.) and a photodiode array detector (PDA, Shimadzu Corp.)

i. Conditions for acidic mobile phase (A)

Mobile phase: acetonitrile/phosphoric acid solution (0.2 %, pH 3.0) (1:9, v/v); column (oven) temperature: 40 °C; flow rate: 1.0 mL/min; detection wavelength: 215 nm.

ii. Conditions for neutral mobile phase (B)

Mobile phase: acetonitrile/phosphate buffer solution (20 mM, pH 6.5) (6:4, v/v); other conditions are the same as described above.

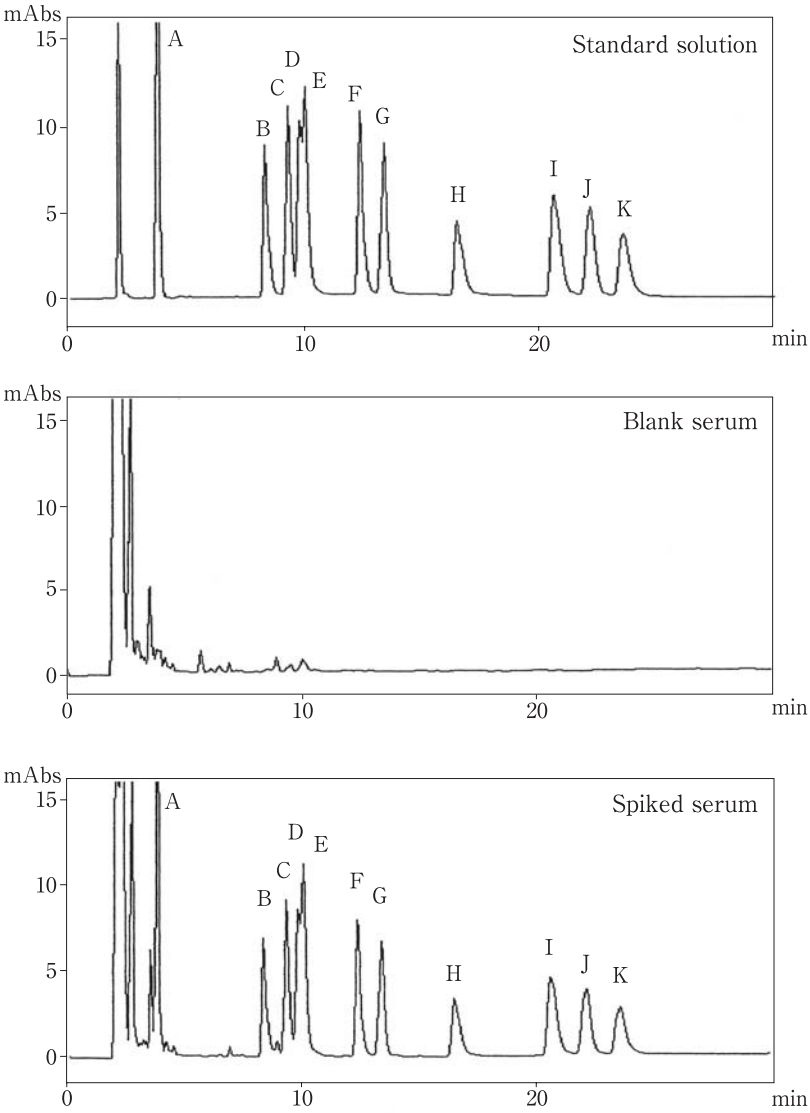
Procedure

i. A 0.5-g (mL) aliquot of a specimen is mixed with 10 μL phosphoric acid in a test tube and mixed well.
ii. The mixture solution is poured into an activated Oasis HLB column[j].
iii. The column is washed with 1 mL of 5 % methanol aqueous solution[k].
iv. A target compound is eluted with 1 mL methanol into a glass vial; the eluate is evaporated to dryness under a stream of nitrogen.
v. The residue is dissolved in 0.5 mL of each mobile phase; a 5-μL aliquot of it is injected into HPLC for analysis.

Assessment of the method

Figure 3.3 shows HPLC chromatograms for tricyclic and tetracyclic antidepressants (5 μg/mL) spiked into human serum using the mobile phase (B). With the use of the Eclipse XDB-C_8 column, the peak top of nortriptyline (D) could be separated from that of amoxapine (E), but major parts of their peaks could not be separated. When the acidic mobile phase (A) was used, the 7 kinds of tricyclic antidepressants could be separated; but mianserin and setiptiline could

Figure 3.3



Chromatograms for tricyclic and tetracyclic antidepressants and carbamazepine obtained by HPLC-UV. A: carbamazepine, B: desipramine, C: maprotiline, D: nortriptyline, E: amoxapine, F: setiptiline, G: mianserin, H: imipramine, I: amitriptyline, J: trimipramine, K: clomipramine.

not be separated from amoxapine. However, the three compounds could be separated using the neutral mobile phase (B) (*Figure 3.3*). Therefore, both mobile phases (A) and (B) should be used according to needs. For tentative identification of drugs, a UV absorbance spectrum can be measured together with the confirmation of coincidence of retention time of a test peak with that of an authentic compound. Although an analytical case using TSK gel Super-Octyl

(particle diameter 2 μm) was reported [3], the separation among desipramine, maprotiline and amoxapine could not be achieved. It is generally difficult to make simultaneous detection of many drugs using a UV detector. In addition, peaks of benzodiazepines sometimes overlap those of the antidepressants. When a UV detector is used, at least two different conditions using different mobile phases or columns should be used. When a mass spectrometer is used as a detector, the reliable identification of a compound is possible without complete separation of two peaks. The detection limit of each antidepressant measured by the present HPLC-UV is about 0.01 μg/g (mL); the toxic and fatal levels of antidepressants can be measured.

The retention time for lofepramine is long; it cannot be eluted under the present HPLC conditions. The ratio of methanol or acetonitrile in a mobile phase should be much higher to enable detection of the peak of lofepramine.

Poisoning cases, and toxic and fatal concentrations

In *Table 3.1*, the therapeutic and toxic blood concentrations of each compound are shown [4]. For all drugs, the toxic levels were in the order of μg/mL. Two examples of poisoning cases are shown below.

Case 1 [5]: A 35-year-old female was found collapsed in the morning at her house by her family member. Although she was sent to a doctor by an ambulance car, she had been dead already. Many empty packages for tablets were discovered in her room; the death due to drug poisoning was suspected. As her past history, hypotension, depression and insomnia were disclosed; antidepressants and antianxiety drugs had been prescribed by a doctor. Among the empty packages, the number of them was largest for Tecipul (setiptiline maleate); as many as 200 tablets of Tecipul were found missing. As results of GC/MS analysis, setiptiline was

Table 3.1

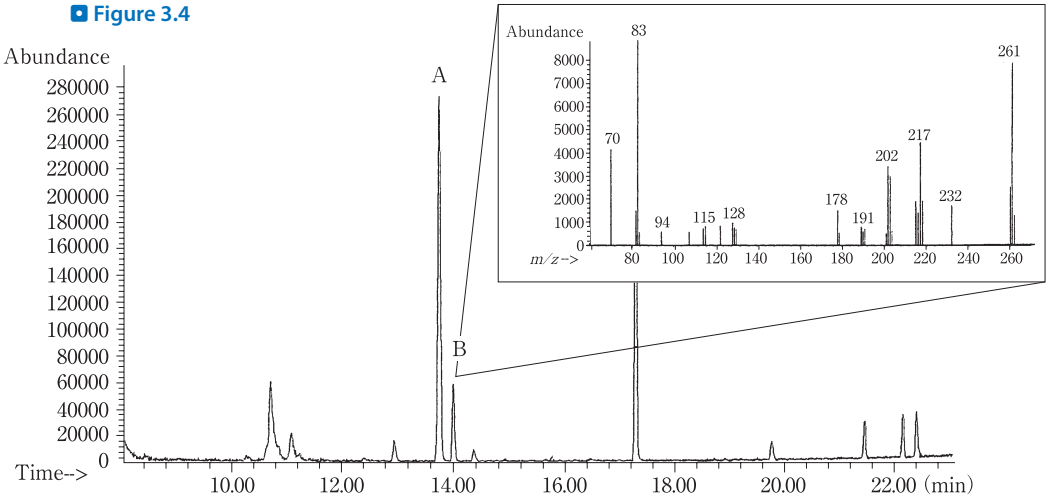
Blood concentrations of tricyclic and tetracyclic antidepressants

Compound	Therapeutic level* (μg/mL)	Toxic level (μg/mL)
amitriptyline	0.038–0.162	0.6–15
amoxapine	0.017–0.093	0.9–20
carbamazepine	1.4–12	12–77
clomipramine	0.10–0.48	0.4–0.54
desipramine	0.016–0.567	0.5–7.8
dosulepin	0.03–0.07	0.3–4.5
imipramine	0.008–0.105	0.3–30
lofepramine	0.04–0.14	–
maprotiline	0.168–0.718	1.3–13
mianserin	0.015–0.070	2.3
nortriptyline	0.022–0.242	0.5–8.4
setiptiline	0.001–0.003	>0.02
trimipramine**	0.008–0.241	0.4–12

* Largely concentrations in blood plasma.

** Now not available for ethical use in Japan.

Figure 3.4



TIC and a mass spectrum obtained from the blood extract of a victim in fatal setiptiline poisoning. A: promethazine (IS); B: setiptiline.

detected from her stomach contents, whole blood and urine; its lood concentrations were 0.78–1.77 μg/mL. In *Figure 3.4*, a TIC and a mass spectrum obtained from blood of this victim are shown.

Case 2 [6]: A 26-year-old female ingested a massive dose of drugs, which had been prescribed by a psychiatrist, in the evening. After about 30 min, ataxia appeared and she fell into sleep; at this time point, she was discovered by her family member. About 2 h after the ingestion, she was sent to an emergency room of a hospital. Her physical conditions at admission were: consciousness level, 300 (Japan Coma Scale, JCS); pupil diameter, 4 mm; light reaction, prompt; heart rate, 100/min with the sinus rhythm. Although gastric lavage and administration of a purgative and activated charcoal were performed after admission, no improvement of her consciousness could be achieved. Next morning, her mother brought empty packages of carbamazepine tablets; she was diagnosed as carbamazepine poisoning. Hemodialysis was performed with careful control of her respiration and circulation, but she died in spite of the intensive care on the second day. The analysis of blood sampled about 15 h after ingestion showed 71.3 μg/mL of carbamazepine, which was a fatal level.

Notes

a) As specimens for analysis, body fluids, such as whole blood, serum (plasma) and urine, can be used. Tablets can be destroyed into powder using a mortar to be extracted with an organic solvent before analysis. For the specimens containing solid particles, such as stomach contents, the liquid-liquid extraction is suitable.

b) As IS, deuterium-labeled compounds, such as imipramine-d_3 (Sigma), are most desirable; but there are problems for easiness of getting them or for their high costs. When a deuterium-labeled compound cannot be used, one of other tricyclic or tetracyclic antidepressants can

Table 3.2

RI values and mass spectra of tricyclic and tetracyclic antidepressants

Name	MW	RI**	Mass fragmentation ions (*m/z*)***				
amitriptyline	227.18	2205 (2196)	58	202	215	91	277
amoxapine	313.10		245	257	193	228	313
carbamazepine	236.10	2285 (2290)	193	236	165		
clomipramine	314.15	2455 (2406)	58	269	85	314	227
desipramine	226.18	2225 (2242)	195	235	208	71	266
dosulepin	295.14	2385 (2380)	58	221	202	295	234
imipramine	280.19	2215 (2223)	58	85	234	193	280
lofepramine [9]	418.18		58	235	193	418	208
maprotiline	277.18	2390 (2356)	59	70	277	203	191
mianserin	264.16	2210 (2211)	193	72	264	165	178
nortriptyline	263.17		202	220	189	91	263
setiptiline [5]	261.15	2255 (2210)	83	261	70	202	217
trimipramine	294.21	2225 (2201)	58	249	193	99	294

*: molecular weight.
**: retention index [7] (from the TIAFT Bulletin [8]).
***: typical mass fragmentation ions [7,9] (underline; base peak ion).

be used as IS, after confirmation of the absence of the compound in the specimen. For such a purpose, retention indexes and mass spectral data are presented in *Table 3.2*.

c) Upon the extraction step, the pH of the aqueous phase should be higher than 11, because extraction efficiencies are markedly decreased below pH 11. Such a phenomenon is observed especially for desipramine, nortriptyline, maprotiline and amoxapine, which have imino groups in their side chain structures. There is also a possibility of low recoveries due to adsorption of drugs to glasswares; this problem can be overcome by siliconizing the glasswares or by adding carriers such as triethylamine.

d) Various reports including liquid-liquid extraction of antidepressants using various organic solvents, such as hexane, ethyl acetate and diethyl ether, were reported. The best solvent can be chosen according to the kind of a target drug.

e) Imipramine and amitriptyline are metabolized into desipramine and nortriptyline, respectively, in a human body. Both metabolites show comparable or even more active pharmacological effects. Therefore, to discuss the relationship between toxicity and the concentration of an antidepressant drug, the combined concentration of the drug itself and its active metabolite should be assessed. For example, when imipramine is ingested, the combined concentration at 1 μg/mL of imipramine plus desipramine is being regarded as the toxic level.

f) The Oasis HLB is a new mixed-type column for solid-phase extraction being sold by Waters. This column has overcome problems of the conventional silica-based packing materials; it uses a porous polymer packing material, and can simultaneously hold both polar and non-polar compounds with high efficiencies. Similar columns are also commercially available from different manufacturers and usable in these experiments. However, if free silanol groups remain in the packing material, the drugs adsorb to the material too firmly, resulting in low recoveries of drugs. The mixed-type solid-phase extraction column with minimal residual free silanol groups should be used.

g) The Milli Q water is the one, which had been passed through a Millipore filter with a special ion-exchanging system, and is being widely used in laboratories. This water is usable for a mobile phase of HPLC in place of distilled water.

h) A mobile phase solution, after suitable mixing with a polar organic solvent, is usually degassed under reduced pressure using an aspirator together with sonication. The glass container should thus be pressure-resistant. The solution should not be left under reduced pressure for a long time, because methanol or acetonitrile is evaporated resulting in changes of composition ratio of the mobile phase.

i) According to the kinds of columns used (manufacturer, type, internal diameter and length), the turn of drugs to be eluted and also the retention time become different. *Table 3.3*

Table 3.3

Differences in retention times according to different HPLC columns

Mobile phase A	pK_a	i	ii	iii	iv
amitriptyline	9.4	1.16	1.16	1.18	1.16
amoxapine	*	0.60	0.60	0.57	0.58
clomipramine	9.5	1.71	1.78	1.89	1.83
desipramine	9.5	0.91	0.92	0.91	0.89
imipramine	9.5	1.00 (11.322 min)	1.00 (11.983 min)	1.00 (17.259 min)	1.00 (17.399 min)
maprotiline	10.5	1.11	1.11	1.11	1.06
mianserin	7.1	0.60	0.61	0.55	0.58
nortriptyline	9.7	1.05	1.06	1.07	1.05
setiptiline	7.8	0.57	0.59	0.54	0.57
trimipramine	7.7	1.30	1.30	1.35	1.31

* No data available

Mobile phase B	pK_a	i	ii	iii	v	vi	vii
amitriptyline	9.4	1.24	1.20	1.32	1.35	1.22	1.24
amoxapine	*	0.60	0.49	0.45	0.45	0.41	0.66
clomipramine	9.5	1.38	1.38	1.92	1.60	1.45	1.45
desipramine	9.5	0.49	0.53	0.52	0.56	0.41	0.53
imipramine	9.5	1.00 (15.195 min)	1.00 (24.237 min)	1.00 (14.003 min)	1.00 (38.997 min)	1.00 (18.687 min)	1.00 (8.869 min)
maprotiline	10.5	0.53	0.60	0.45	0.56	0.41	0.53
mianserin	7.1	0.80	0.78	1.27	0.49	0.64	0.96
nortriptyline	9.7	0.58	0.62	0.62	0.67	0.51	0.59
setiptiline	7.8	0.75	0.70	1.06	0.49	0.59	0.86
trimipramine	7.7	1.34	1.28	1.60	1.20	1.20	1.49

Column i) Eclipse XDB-C_8 (250 × 4.6 mm i. d.)
Column ii) Inertsil ODS-2 (250 × 4.6 mm i. d.)
Column iii) Develosil UG-5 (250 × 4.6 mm i. d.)
Column iv) TSK gel OSD-80T_M (250 × 4.6 mm i. d.)
Column v) TSK gel OSD-80T_M (150 × 4.6 mm i. d.)
Column vi) TS Kgel OSD-80T_S (150 × 4.6 mm i. d.)
Column vii) Discovery C_{18} (150 × 4.6 mm i. d.)
The relative retention time values were calculated by assuming that of imipramine as 1.00.

shows the results on relative retention times for ten antidepressant drugs using different types of HPLC columns. Especially, the residual silanol groups remaining in the column cause longer retention times and broadening of the peak width. To overcome these problems, the ratio of methanol or acetonitrile in the mobile phase is increased; the addition of 10–20 mM counter ions, such as triethylamine, is also effective to some extent.

j) The flow rate for the solid-phase column upon adsorption, washing and elution should be 1–2 mL/min. Upon washing the column, too slow flow rate causes the elution of a target drug. It should be cautioned that the column should not be dried up just after its activation; however, just before elution of a target drug, the column should be dried up. The analysts should be careful not to make a mistake on the above matter. In this section the most common procedure is presented, but a different washing and elution procedure using a different solvent can be used.

k) The column washing should be sufficient. If not, protein components cannot be removed; upon elution with 100 % methanol, proteins appear together with a drug in the eluate. In such a case, the removal of proteins by filtration becomes necessary.

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