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Forensic analysis of eleven cyclic antidepressants in human biological samples using a new reversed-phase chromatographic column of 2 μm porous microspherical silica gel

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Abstract

A high-performance liquid chromatographic method has been developed for the forensic analysis of eleven frequently used cyclic antidepressant drugs (ADSs) (amitriptyline, amoxapine, clomipramine, desipramine, dosulepine, doxepin, imipramine, maprotiline, melitracen, mianserine and nortriptyline) using a recently developed reversed-phase column with 2 μm particles for the analysis of biological samples. The separation was carried out using two different C_8 reversed-phase columns (column 1: 100 mm×4.6 mm I.D., particle size 2 μm, TSK gel Super-Octyl; column 2: 100 mm×4.6 mm I.D., particle size 5 μm, Hypersil MOS-C_8) for comparison. The mobile phase was composed of methanol–20 m*M* KH_2PO_4 (pH 7) (60:40, v/v) and the flow-rate was 0.6 ml/min for both columns. The absorbance of the eluent was monitored at 254 nm. When the eleven drugs were determined, the sensitivity with the 2 μm particles was about five times greater than with the 5 μm particles. Retention times on column 1 were shorter than those on column 2. These results show that the new ODS column packing with a particle size of 2 μm gives higher sensitivity and a shorter analysis time than the conventional ODS column packing when applied to the analysis of biological samples.

Keywords: Forensic toxicology; Cyclic antidepressant; Amitriptyline; Amoxapine; Clomipramine; Desipramine; Dosulepine; Doxepin; Imipramine; Maprotiline; Melitracen; Mianserine; Nortriptyline

1. Introduction

The use of cyclic antidepressants (ADSs), such as amitriptyline, amoxapine, clomipramine, desipramine, dosulepine, doxepin, imipramine, maprotiline, melitracen, mianserine and nortriptyline, is becoming increasingly prevalent for the treatment of depression and these drugs are frequently encountered in emergency toxicology screening, drug-abuse testing and forensic medical examinations [1]. In addition, reports of ADSs concentrations in fetal overdose cases are increasing [2–10]. Several high-performance liquid chromatographic (HPLC) methods have already been reported for the simultaneous liquid chromatographic analysis of ADSs and/or their major metabolites in blood [11–20]. However, no report has been published on the analysis of a

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large number of ADSs in human tissues (blood, urine, brain and liver).

Recently, a new reversed-phase chromatographic column, TSK gel Super-Octyl, based on 2 μm silica gel, became commercially available from Toshoh (Tokyo, Japan) [21]. This column showed fewer ionic and metal interactions than previous columns. In addition, the use of the smaller particle size gives a higher column efficiency than other reversed-phase columns for many compounds. Therefore, faster separation and better resolution can be achieved on Super-Octyl. A few factors must be considered, however, when using this column: the retention capacity is lower than that of other conventional ODS columns so that the content of organic modifier in the mobile phase should be lowered, and the void volumes in the operating system must be reduced to a minimum.

In this paper, we report the forensic analysis of eleven cyclic ADSs in human biological samples using this reversed-phase column in comparison with a conventional column.

2. Experimental

2.1. Reagents

Amitriptyline, amoxapine, dosulepine, imipramine, lofepramine, melitracen, mianserine, nortriptyline, trimipramine and sulpiride were gifts from Yamanouchi (Tokyo, Japan), Lederle Japan (Osaka, Japan), Kaken Kagaku (Kyoto, Japan), Yoshitomi (Osaka, Japan), Daichi (Tokyo, Japan), Takeda (Osaka, Japan), Sankyo (Tokyo, Japan), Dainihon (Osaka, Japan), Shionogi (Osaka, Japan) and Fujisawa (Osaka, Japan), respectively. Clomipramine, desipramine and maprotiline were gifts from Ciba-Geigy (Rueil-Malmaison, France). Doxepin was purchased from Sigma (St. Louis, MO, USA). Diazepam, *n*-hexane and 3-methyl-1-butanol (isoamyl alcohol) were purchased from Wako (Tokyo, Japan). The mobile phase was prepared by mixing deionized water obtained using a Milli-Q system (Millipore, Bedford, MA, USA) and HPLC-grade organic solvent. Phosphate buffer was made from NaH_2PO_4 (adjusted to pH 7 with 1 *M* NaOH).

2.2. Biological samples

Human tissue samples were obtained at autopsy and kept at −20°C until analysis. Drug-free human tissues were used as control samples.

2.3. Standard solutions and calibration

A stock standard solution containing eleven ADSs (1 mg/ml of each compound in methanol) was stable for at least 3 months when stored at −20°C. Spiked serum, urine and tissue samples were prepared at concentrations of 0.05, 0.5 and 5 μg/ml of each compound by diluting appropriate volumes of stock solution. The parameters for calibration curves were obtained by linear regression of the peak-height ratios versus concentration.

2.4. Chromatography

The HPLC equipment consisted of a pump (Model CCPS, Tosho, Tokyo, Japan) and a variable-wavelength UV detector (Model UV-8000, Tosho). The separation was achieved using a C_8 reversed-phase column (column 1: 100 mm×4.6 mm I.D., particle size 2 μm, TSK gel Super-Octyl; column 2: 100 mm×4.6 mm I.D., particle size 5 μm, Hypersil MOS-C_8, Yokogawa, Tokyo, Japan). The mobile phase was composed of methanol–20 m*M* KH_2PO_4 (pH 7) (60:40, v/v) and the flow-rate was 0.6 ml/min for both columns. The absorbance of the eluent was monitored at 254 nm. The procedure was performed at ambient laboratory temperature (approx. 23°C).

2.5. Extraction procedure

2.5.1. Blood and urine

We added 100 μl internal standard (diazepam, 2 μg/ml), 200 μl 20% Na_2CO_3, 0.5 ml water and 3 ml *n*-hexane–isoamylalcohol (98.5:1.5, v/v) to 0.5 ml blood (serum) or urine (or standard aqueous solution) in 15 ml teflon-lined screw-capped culture tubes. After mixing for 2 min, the tubes were centrifuged at 1200 *g* for 5 min. The organic phase (about 2.5 ml) was transferred to a clean conical tube and evaporated in a water bath at about 40°C under a gentle stream of nitrogen. The residue was dissolved in 100

μl mobile phase and 10 μl was injected into the HPLC apparatus.

2.5.2. Brain and liver

The tissues (1 g) were homogenized in a mixture of 9 ml of 0.1 *M* HCl and 100 μl internal standard (diazepam, 20 μg/ml), and then centrifuged at 15 000 *g* for 10 min. The supernatant (1 ml) was 0.5 ml 20% Na_2CO_3 and 4 ml *n*-hexane–isoamylalcohol (98.5:1.5 v/v). After mixing for 5 min, the tubes were centrifuged at 1200 *g* for 5 min. The organic phase was transferred to a clean conical tube and evaporated in a water bath at about 40°C(under a gentle stream of nitrogen. The residue was dissolved in 100 μl mobile phase and filtered by microconcentrator (microcon-30, Grace Japan, Tokyo, Japan). A 10 μl volume was injected into the HPLC apparatus.

2.6. Accuracy and recovery

The accuracy and the recovery were calculated by comparing the peak heights of the eleven ADSs (0.05 and 0.5 μg/ml or g) in spiked samples after extraction from serum, urine or tissue samples with the peak heights of a series of unextracted reference standards.

3. Results and discussion

3.1. Retention times

Fig. 1 shows an HPLC chromatogram for the spiked biological samples using column 1. No interfering peaks were observed in the blank samples. The eleven ADSs and the internal standard were well separated. Retention times on column 1 in human sera were shorter than those on column 2 (Table 1). Under the optimized chromatographic conditions described, all drugs eluted within 25 min. Lofepramine, sulpiride and trimipramine were not detected by this method and the retention time of amoxapine, desipramine and maprotiline was very similar because the determination of these drugs requires a different elution system. These results show that the retention times of ADSs depend mainly on the particle size of the reversed-phase column.

No interfering peaks appeared when the following drugs were added to serum: barbital, hexobarbital, pentobarbital, trimethadione, ethosuximide, primidone, phenobarbital, carbamazepine and phenytoin.

Table 1
Retention times of eleven cyclic antidepressants in human biological samples by the present HPLC method using columns 1 and 2

Drug	Retention time (min)	
	Column 1[a]	Column 2[b]
1. Amitriptyline	16.2	28.5
2. Amoxapine[c]	5.6	7.8
3. Clomipramine	20.6	40.2
4. Desipramine[c]	5.6	7.8
5. Dosulepine	10.6	19.7
6. Doxepine	8.5	10.3
7. Imipramine	12.8	23.8
8. Maprotiline[c]	5.6	7.8
9. Melitracen	19.1	33.8
10. Mianserine	9.3	13.5
11. Nortriptyline	7.0	9.2

[a] Column 1: 100 mm×4.6 mm I.D., particle size 2 μm (TSK gel Super Octyl).
[b] Column 2: 100 mm×4.6 mm I.D., particle size 5 μm (Hypersil MOS-C_8).
Diazepam was added as internal standard.
Mobile phase, methanol–20 m*M* KH_2PO_4 (pH 7) (60:40, v/v); flow-rate, 0.6 ml/min; detection wavelength, 254 nm for the both columns. All instruments and the column were operated at ambient laboratory temperature (ca. 23°C).
[c] The retention time of amoxapine, desipramine and maprotiline was very similar.

3.2. Limits of quantification

The limit of quantification using column 1 is the lowest concentration on the standard curve which can be measured with acceptable accuracy (coefficient of variation, C.V.<20%). The limit of quantification in human sera and urine was 0.05 μg/ml for the eleven ADSs (Table 2). On the other hand, that of other samples was 0.5 μg/ml. The sensitivity for the eleven ADSs was about five times better on column 1 than on column 2. This method produced approximately 2–20 times higher sensitivity compared with the traditional methods that have been reported involving detection at 254 nm [11,13–15,17,19,20]. These quantification limits are adequate for forensic and clinical analysis [1]. Some reports have involved detection at 245 nm [13,14,17] or close to this [11,15,20], but there is less interfer-

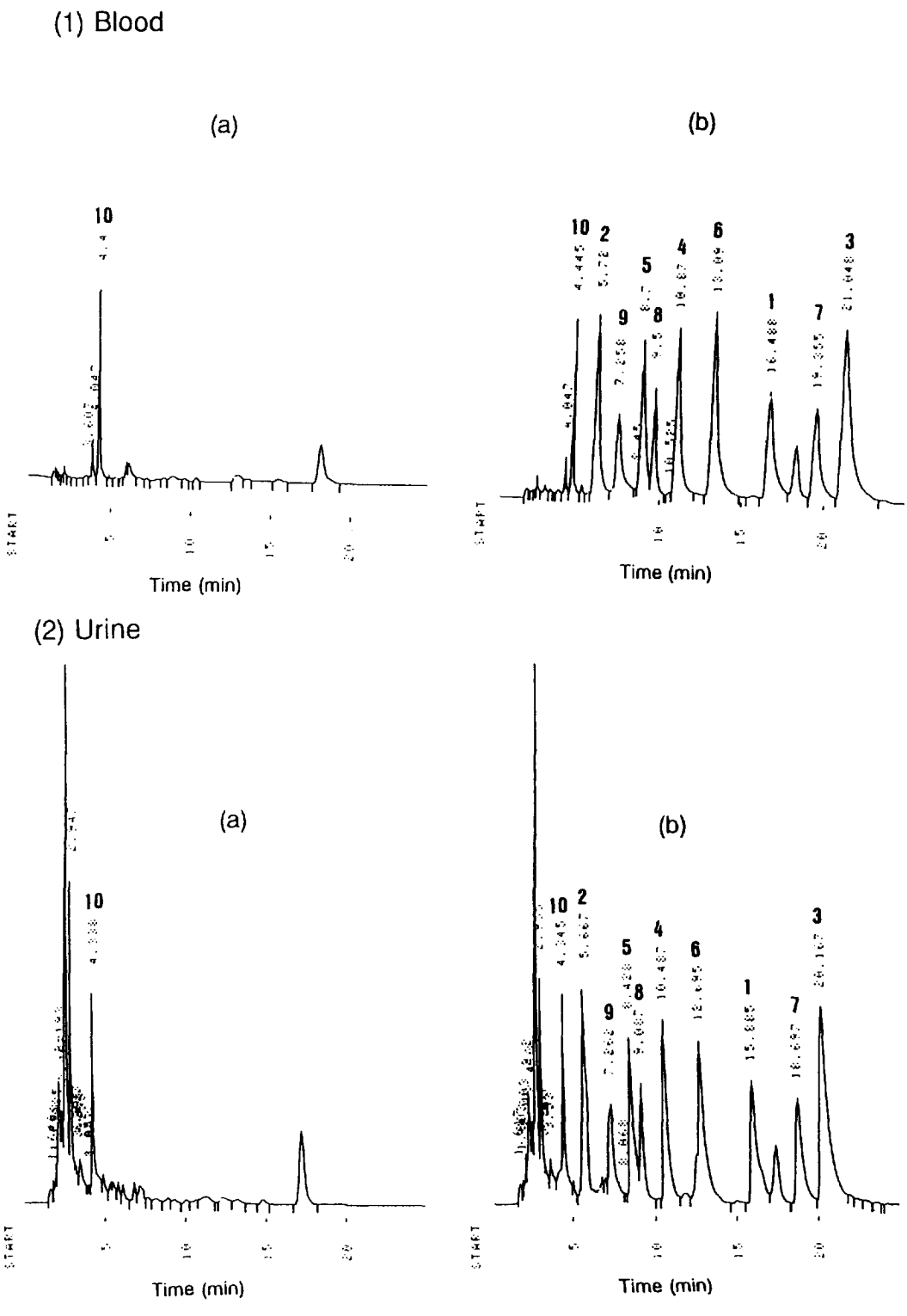

Fig. 1. Chromatograms of eleven cyclic antidepressants in (1) serum, (2) urine, (3) brain and (4) liver using column 1. (a) Drug-free sample; (b) drugs added to the drug-free sample. The drug concentrations of the eleven cyclic antidepressants was 0.5 μg/ml. 1=Amitriptyline; 2=Amoxapine; 3=Clomipramine; 4=Dosulepine; 5=Doxepine; 6=Imipramine; 7=Melitracen; 8=Mianserin; 9=Nortriptyline; 10=Diazepam (internal standard). The peaks of desipramine and maprotiline overlapped that of amoxapine.

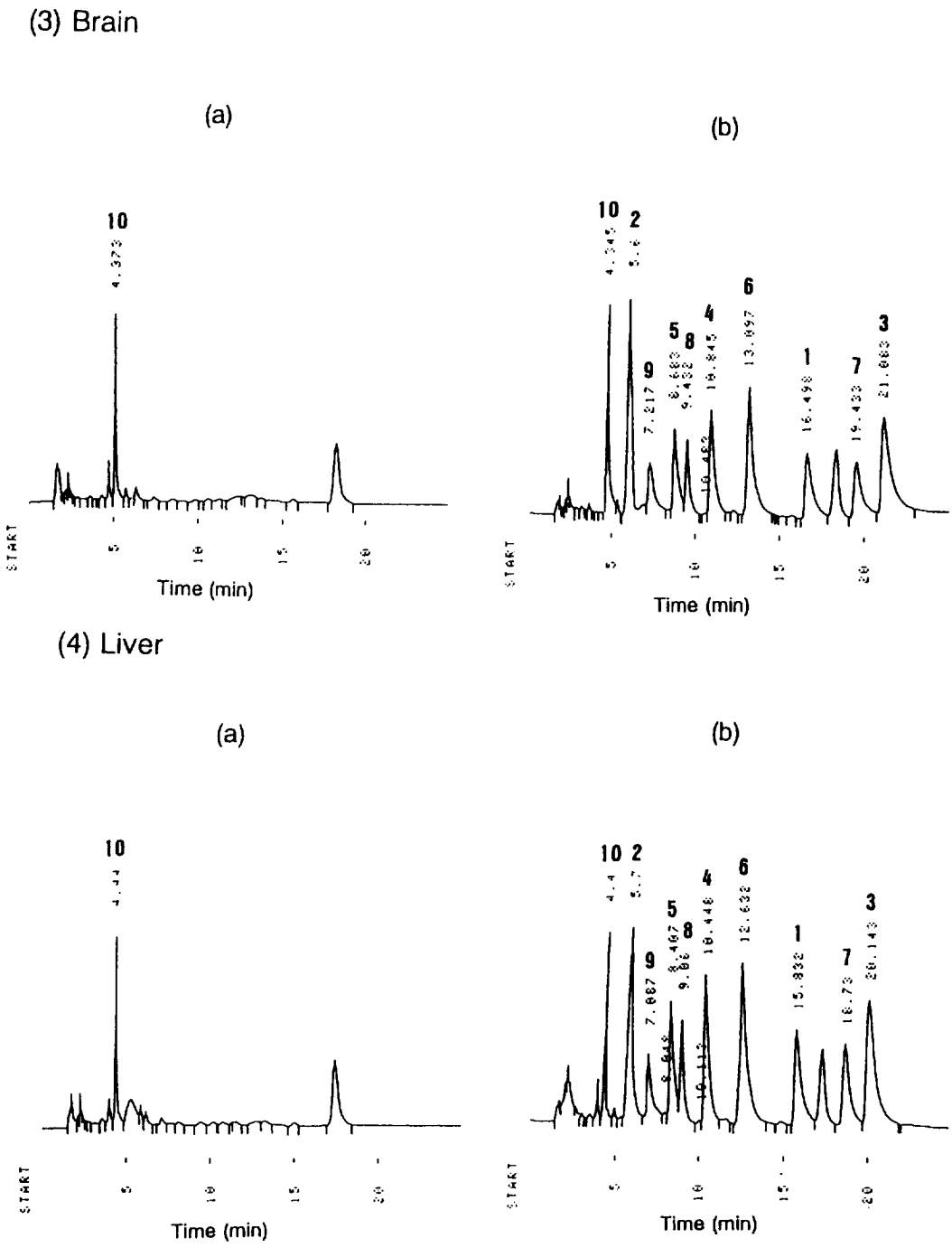

Fig. 1. (*continued*)

Table 2
Precision study of eleven cyclic antidepressants in human sera by the present HPLC method (column 1)[a]

Drug	Added (μg/ml)	Mean (μg/ml)	Within-day C.V.(%)	Between-day C.V.(%)
1. Amitriptyline	0.05	0.051	5.9	6.0
	0.5	0.51	3.9	5.9
	5	5.2	1.9	5.8
2. Amoxapine[a]	0.05	0.049	4.1	6.1
	0.5	0.48	4.2	6.3
	5	5.1	2.0	3.9
3. Clomipramine	0.05	0.049	4.1	6.1
	0.5	0.52	1.9	3.8
	5	5	2.0	4.0
4. Desipramine[a]	0.05	0.049	4.1	6.1
	0.5	0.49	3.9	4.1
	5	5.1	2.6	3.9
5. Dosulepine	0.05	0.048	4.8	6.3
	0.5	0.49	4.2	6.1
	5	4.9	4.1	6.1
6. Doxepine	0.05	0.049	3.2	6.1
	0.5	0.52	3.8	3.8
	5	5.1	3.9	3.9
7. Imipramine	0.05	0.049	4.1	6.1
	0.5	0.51	3.9	5.9
	5	4.9	1.2	4.1
8. Maprotiline[a]	0.05	0.048	4.2	6.3
	0.5	0.49	2.0	4.1
	5	5.1	2.4	3.9
9. Melitracen	0.05	0.051	3.6	5.9
	0.5	0.51	2.0	5.9
	5	5	1.5	4.0
10. Mianserine	0.05	0.051	3.9	6.0
	0.5	0.52	2.7	3.8
	5	5	1.2	4.0
11. Nortriptyline	0.05	0.051	3.9	5.9
	0.5	0.49	4.1	6.1
	5	5	1.4	4.0

$n=5$, C.V.=coefficient of variation.
[a] The retention time of amoxapine, desipramine and maprotiline was very similar. Therefore, the precision and accuracy of each drug was determined separately.

ence from other column packing when the range of wavelength is greater.

3.3. Precision and accuracy

The precision and accuracy using column 1 with human sera is also shown in Table 2. Within-day reproducibility was assessed using five samples at three different concentrations and analyzed on the same day. The within-day C.V. varied from 1.2 to 5.9%, and the between-day C.V. was found to range 3.8 to 6.3%. The precision and accuracy of other samples were similar to those of human sera (data not shown).

3.4. Recovery

Six liquid–liquid extraction solvents were investigated, including butylchloride [11], diethyl ether–chloroform (80:20, v/v) [13], diethyl ether [14], 1-chlorobutane–isoamyl alcohol (98.5:1.5, v/v) [15], *n*-hexane [17] and *n*-hexane–isoamylalcohol (98.5:1.5, v/v). The mixture 1.5% isoamyl alcohol–*n*-hexane was chosen as the liquid–liquid extraction

Table 3
Relative recovery of eleven cyclic antidepressants added to drug-free human sera by the present HPLC method (column 1)[a]

Drug	Actual amount added (μg/ml)	Mean recovery (%)
1. Amitriptyline	0.05	97
	0.5	99
2. Amoxapine[a]	0.05	103
	0.5	102
3. Clomipramine	0.05	97
	0.5	95
4. Desipramine[a]	0.05	94
	0.5	97
5. Dosulepine	0.05	98
	0.5	103
6. Doxepine	0.05	96
	0.5	99
7. Imipramine	0.05	101
	0.5	102
8. Maprotiline[a]	0.05	98
	0.5	96
9. Melitracen	0.05	97
	0.5	96
10. Mianserine	0.05	98
	0.5	105
11. Nortriptyline	0.05	96
	0.5	102

$n=5$. Relative recovery=(actual level/expected level)×100.
[a] The retention time of amoxapine, desipramine and maprotiline was very similar. Therefore the relative recovery of each drug was determined separately.

solvent because it produced a better extraction than the other solvents (data not shown).

To eleven drug-free serum samples were added 0.05 and 0.5 μg/ml of each drug. Relative recoveries using the internal standard were calculated by comparing the values obtained for spiked serum with those actually added. The recovery in human sera ranged from 94 to 103% for all drugs (Table 3). The C.V. using column 1 was 2.7 to 6.6%. The recovery in other samples were approximately the same as those of human sera (data not shown).

3.5. Linearity

The calibration curves (the ratio between the peak-height of the drugs analyzed and that of the internal standard versus the concentration of each drug) exhibited linearity over the concentration range 0.05–5 μg/ml serum. The equations and r values for the curves were: $y=0.3x+0.02$, $r=0.998$ for amitriptyline; $y=0.52x-0.3$, $r=0.996$ for amoxapine; $y=0.36x+0.17$, $r=0.999$ for clomipramine; $y=0.5x+0.14$, $r=0.998$ for desipramine; $y=0.49x-0.01$, $r=0.998$ for dosulepine; $y=0.47x+0.02$, $r=0.998$ for doxepine; $y=0.46x-0.03$, $r=0.998$ for imipramine; $y=0.52x-0.05$, $r=0.999$ for maprotiline; $y=0.23x+0.09$, $r=0.999$ for melitracen; $y=0.27x+0.07$, $r=0.999$ for mianserine; $y=0.25x-0.05$, $r=0.998$ for nortriptyline. The r values of other samples were similar to those of human sera (data not shown).

HPLC is widely used in routine applications owing to its sensitivity, specificity and low cost. Even although these drugs are never administered together, the availability of a single chromatographic method, suitable for their simultaneous detection, is very useful in the forensic laboratory, as the maintenance of separate chromatographic methods for each drug would be more expensive.

A column packing with a particle size of 2 μm (TSKgel Super-ODS) and pore volume and specific surface area about one third of those of the conventional column packing has recently been developed [21]. This ODS column packing has the following advantages: (1) more rapid determination can be expected at room temperature, even without gradient elution compared with the conventional column packing, (2) the amount of organic solvent required is smaller and (3) more sensitive determination is possible.

The detection and determination of ADSs with a new ODS column packing with a particle size of 2 μm was more sensitive, provided better separation and was more rapid than with the conventional ODS column packing. The rapid turnaround time and accuracy of this method make it suitable for emergency and forensic toxicology as well as clinical medicine, allowing the rapid detection, confirmation and quantification of many ADSs using a single method.

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