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Dual ultraviolet wavelength high-performance liquid chromatographic method for the forensic or clinical analysis of seventeen antidepressants and some selected metabolites

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ABSTRACT

A sensitive method suitable for the determination of tricyclic and other antidepressants in postmortem and clinical specimens is presented. The procedure, which utilizes reversed-phase HPLC combined with dual ultraviolet wavelength detection, enables the separation of 17 commonly prescribed antidepressants and some selected metabolites in a single extraction. Peak purity was confirmed using absorbance ratios at 220 nm and 254 nm wavelengths and revealed little interference from other eluting analytes. The blood detection limit for most antidepressants was 50 ng/ml. The most commonly observed antidepressants in 281 forensic cases analysed over a two-year period with the described method were dothiepin, amitriptyline, nortriptyline and doxepin.

INTRODUCTION

Depression is one of the most frequent conditions presented in medical and psychiatric practices today. Imipramine, amitriptyline and dothiepin are among the more common older tricyclic antidepressants (TCA), whilst mianserin, maprotiline, fluoxetine and reversible monoamine oxidase inhibitors such as moclobemide are now also being used in psychiatric medicine.

The incidence of antidepressants in sudden deaths submitted for toxicological analysis may be as high as 12% [O. H. Drummer; unpublished observations]. Consequently the need for an analytical procedure capable of both identifying and quantitating antidepressants is important in both clinical and forensic toxicology.

Screening methods available for monitoring antidepressants include radioimmunoassay [1], enzyme-immunoassay [2] and fluorescence polarization [3]. These methods are capable of identifying the presence of antidepressants, but generally are unable to discriminate the parent drugs from their metabolites and often have poor sensitivity at therapeutic concentrations. These methods also require serum/plasma, or urine, and may not be suitable for analysis of whole blood specimens; a significant drawback for forensic toxicology. Thin layer chromatography has also been used [4], but is only useful for urine due to the lack of sensitivity. Gas chromatographic (GC) methods have been demonstrated as sensitive for most antidepressants, however there are difficulties in separating all of the TCA in one run [5–7]. HPLC methods for the determination of antidepressants have proven more popular than GC in

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recent years partly due to the easier availability and low cost of sensitive ultraviolet (UV) absorbance detectors. Although early methods utilized fixed wavelength detectors at a wavelength of 254 nm, improved sensitivity has been reported at lower wavelengths; *e.g.* 210, 215, 220, 240, 242 and 245 nm (see ref. 5 for review). One report has utilized simultaneous detection of 214 and 254 nm, using comparison of the ratio of absorbance at the two wavelengths to detect possible chromatographic interference of imipramine with fluoxetine [8].

Since there is no published method which separates all antidepressants, we have developed a rapid, simple isocratic HPLC method using dual ultraviolet wavelength detection capable of detecting 17 antidepressants and a number of selected metabolites. Although this analytical approach is novel for antidepressants, dual ultraviolet wavelength HPLC methods have been previously described for the analysis of barbiturates [9]. The described method is suitable for the analysis of antidepressants at therapeutic concentrations in post-mortem blood, other tissues and ante-mortem serum/plasma, and has very little interference from other commonly encountered drugs.

EXPERIMENTAL

Materials

Acetonitrile and hexane were of HPLC grade (Mallinckrodt, Melbourne, Australia) and diethylamine was Labgrade (Fluka, Melbourne, Australia). All other chemicals were of Analytical Reagent grade (Ajax, Melbourne, Australia). Antidepressants were purchased from Sigma (St. Louis, MO, USA), provided by the Curator of Standards at the Australian Government Analytical Laboratories, or were a gift from the principal drug company: dothiepin, dothiepin sulfoxide HCl and northiaden HCl (Boots, Nottingham, UK); fluoxetine, norfluoxetine maleate (Lilly, Indianapolis, IN, USA); Cianopramine (Roche Pty, Sydney, Australia). Subtilisin (Protease Type V111) was purchased from Sigma.

Reagents and standards

Drug stock solutions (1 mg/ml) were prepared in methanol, stored at −20°C until use, and proved stable for at least one month. Working standards were prepared from stock solutions in whole blood (or appropriate tissue homogenate) to give concentrations from 0.20 to 2.5 mg/l. Whole blood, used in the preparation of standards, was expired donor blood collected by the Red Cross Blood Bank (South Melbourne, Australia).

Chromatographic conditions

The HPLC system consisted of an LC-6 constant flow pump, a SIL-6B autoinjector and an SPD-10A dual wavelength variable spectrophotometric detector which were coupled by a programmable system controller (Shimadzu Oceania, Melbourne, Australia). Data were collected on a multi-functional data processor with built-in thermal plotter and a floppy disk drive storage facility (C-R4A Chromatopac, Shimadzu Oceania).

The detector was operated at wavelengths of 220 nm and 254 nm, and at sensitivities of 0.04 and 0.01 AUFS, respectively. Chromatographic separation was achieved with a Spheri-5 RP-18 column (100 mm × 4.6 mm I.D.; 5 μm; Brownlee insert column fitted into the appropriate column holder) protected by a RP-18 Newguard cartridge (15 mm × 3.2 mm I.D.; 7 μm) (Applied Biosystems, Melbourne, Australia).

The mobile phase consisted of acetonitrile–0.1 *M* sodium dihydrogen phosphate–diethylamine (40:57.5:2.5, v/v/v) (pH 8.0). The flow-rate was 2.0 ml/min at ambient temperature, and the total run time was 40 min.

Extraction procedure

Blood (or serum) standards, controls or unknowns (1.0 ml), and 1.0 μg of cianopramine (internal standard) were added to 10-ml silanized glass extraction tubes. One ml of deionized water was added and the tubes were vortex-mixed before addition of 1 ml 0.2 *M* Na_2CO_3. Tubes were again vortex-mixed and 6 ml of hexane–butan-1-ol (95:5, v/v) were added and the tubes gently agitated for 30 min. Centrifugation (2500 *g*) for 5 min was followed by the transfer of the organic layer to a clean set of silanized extraction

tubes containing 100 μl of 0.2% phosphoric acid. These were gently agitated for 30 min and then centrifuged for 5 min. The organic layer was aspirated and an aliquot of the aqueous layer (30 μl) was injected into the chromatographic system.

Antidepressants can also be extracted from liver homogenates with minor alterations to the above method for blood or serum. A liver homogenate was prepared by homogenizing 10 g of freshly minced liver in 10 ml of deionized water. The pH was adjusted to 10 using 1 *M* NaOH, subtilisin (10 mg) was added and then the homogenate incubated for 60 min at 55°C. The pH was finally adjusted to 7.0 ± 0.5 with dilute mineral acid. Homogenates were either used immediately or stored at −20°C until analysis. Briefly, 0.5 ml of the liver homogenate was added to 10-ml silanized glass tubes, followed by 10 μg of cianopramine (internal standard), 500 μl 2% sodium tetraborate and 8 ml of hexane–butan-1-ol (95:5, v/v). The tubes were gently agitated then centrifuged for 5 min and the organic layer transferred to clean tubes containing 400 μl 0.2% phosphoric acid. Further agitation was followed by centrifugation and removal of the aqueous layer (30 μl) for injection on to the HPLC.

Calculations

Relative retention times (*RRT*) were calculated from chromatograms of unextracted standards prepared in 0.2% phosphoric acid by comparison of each compound's retention time to that of the internal standard. Following an initial identification of the extracted antidepressant based on the *RRT*, a calculation of the ratio of absorbance at the two wavelengths was made to confirm the initial identification and to check peak purity. The peak height was then divided by the peak height of the internal standard. Peak heights at either 220 or 254 nm can be used for these calculations. Concentrations were calculated by comparing the peak-height ratio from samples to that obtained from the corresponding standards containing known amounts of the drug. Generally, four standards were used to produce a standard curve for each drug or metabolite. Linear regression analyses of the standard curves were made using "cricket graph" (Apple MacIntosh Business Systems Inc).

Capacity factors (k') were calculated using the equation: $k' = (t_1 - t_0)/t_0$; where t_1 = retention time of the TCA; and t_0 is the hold up time of the column.

RESULTS AND DISCUSSION

Post-mortem blood samples were assayed in batches incorporating eight standards each containing several drugs at concentrations ranging from 0.20 to 2.5 mg/l. Similarly, other biological fluids or tissue extracts were analysed with the standards prepared in the approriate mix. Each assay also included quality control samples, both external and in-house. An external control, Therapeutic Drug Monitoring (TDM) (level III, Bio-Rad, Australia) contained amitriptyline, nortriptyline, imipramine and desipramine. In-house controls were prepared at two concentrations containing nortriptyline, doxepin, dothiepin, imipramine and amitriptyline. These controls were used to assess precision of the assay procedure. Both within-run and day-to-day precision data, presented in Table I, demonstrate a relative standard deviation of less than 17%, and generally less than 10%.

The conditions described allowed separation of all commonly encountered antidepressants (see Fig. 1). The *RRT* was reproducible allowing all antidepressants to be identified based on *RRT* to the internal standard. Throughout the assay, *RRT* variability was within ±0.03 even for late eluting compounds like amitriptyline, however *RRT* was generally within ±0.01. Standards and unextracted retention time markers (mixes) were interspaced with unknown samples throughout each assay to keep a check on relative retention time.

Although a few compounds were found to elute closely with others, comparison of individual ratios of absorbance from the two wavelengths were used to assist in the identification of antidepressants. For example, amitriptyline (*RRT* = 2.52), trimipramine (*RRT* = 2.45) and mianserin (*RRT* = 2.20) elute relatively closely. Each compound could be easily identified by its individual

TABLE I

QUALITY CONTROL DATA

Drug	Concentration added (mg/l)	Concentration found (mean ± S.D.) (mg/l)	R.S.D. (%)
Within-assay (intra-assay) reproducibility (n = 7)			
Nortriptyline	0.18	0.18 ± 0.01	5.6
	0.88	0.86 ± 0.07	8.1
Doxepin	0.18	0.18 ± 0.03	17
	0.88	0.85 ± 0.03	3.5
Dothiepin	0.18	0.22 ± 0.03	14
	0.90	0.92 ± 0.03	3.3
Imipramine	0.18	0.19 ± 0.02	10
	0.89	0.89 ± 0.03	3.4
Amitriptyline	0.18	0.22 ± 0.01	4.5
	0.88	0.94 ± 0.05	5.3
Between-assay (inter-assay) reproducibility (n = 7)			
Nortriptyline	0.24	0.22 ± 0.02	9.1[a]
	0.88	0.79 ± 0.05	6.3
Doxepin	–	–	–
	0.88	0.82 ± 0.06	7.3
Dothiepin			–
	0.90	0.82 ± 0.03	3.7
Imipramine	0.21	0.21 ± 0.01	4.8[a]
	0.89	0.82 ± 0.03	3.7
Amitriptyline	0.24	0.20 ± 0.03	15[a]
	0.88	0.81 ± 0.05	6.2
Desipramine	0.24	0.22 ± 0.02	9.1[a]

[a] $n = 5$, TDM III external control.

wavelength ratio; amitriptyline 2.6; trimipramine 1.7; and mianserin 3.8. These individual wavelength ratios have been demonstrated to be reproducible from assay to assay with less than 5% variation (see Table II). A list of retention times (*RT*), *RRT* and wavelength ratios of routinely analysed antidepressants and metabolites are shown in Table II. A further list of antidepressants and metabolites and some other psychoactive compounds which extract and elute with the chromatographic conditions described, are shown in Table III. It should be stated, however, that variation in such data may exist between laboratories. Differences in HPLC columns, wavelength calibration, slit-width and noise can all effect *RT*, *RRT* and absorbance ratios. Consequently, these value should be establised in each laboratory proposing to apply the described method.

The analytical method described was suitable for most antidepressants, however, the late eluting compounds particularly amitriptyline, clomipramine and trimipramine may benefit from a gradient HPLC system. Use of such a gradient elution system would increase the sensitivity of these late eluting compounds and may also improve the detection limits, but may reduce the peak separation of other compounds as well as increasing the overall time of analysis. The benefits of isocratic elution, in our opinion, outweighed the requirement of a gradient system. Despite relatively large capacity factors (k') for a

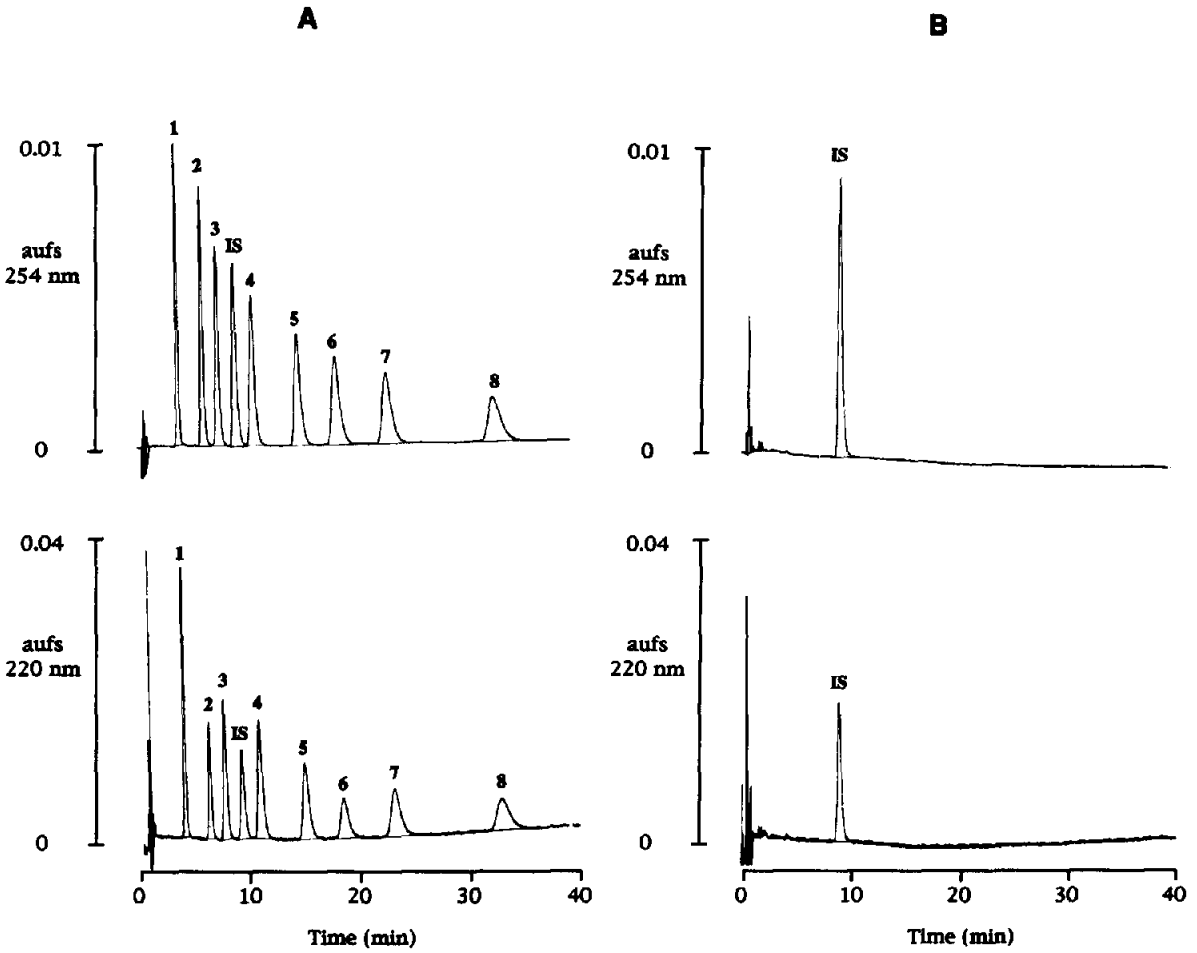

Fig. 1. Chromatograms from analyses at 254 nm in the upper channel, and 220 nm in the lower channel. (A) Unextracted retention marker (Mix A), where: (1) nordoxepin, (2) desipramine, (3) nortriptyline, (4) doxepin, (5) dothiepin, (6) imipramine, (7) amitriptyline, (8) clomipramine, and I.S. = internal standard. (B) Extracted blank blood, where I.S. = internal standard.

number of antidepressants (Table II), the current procedure has demonstrated good separation of all the antidepressants and metabolites listed, and many other commonly prescribed medications (see Tables II and III).

The limit of detection in blood was determined by estimating the minimum concentration equivalent to or greater than three times the background noise (Table IV). These detection limits are suitable for most clinical and forensic analyses of antidepressants from the low steady-state concentration range to the potentially toxic range.

Extraction recovery was determined by comparing the representative peak heights of extracted blood (or liver homogenate) with peak heights of standards prepared in 0.2% phosphoric acid, and is a direct measure of the recovered drug in the final extract. These data (Table IV), show good recovery of routinely assayed antidepressants, most being greater than 60% for blood, and greater than 40% for liver homogenates.

Linearity was studied from five points over the concentration range of 0.20 to 2.5 mg/l in blood. All compounds demonstrated a linear correlation greater than or equal to $r^2 = 0.99$, showing good

TABLE II

RETENTION TIMES, RELATIVE RETENTION TIMES, CAPACITY FACTORS AND DUAL WAVELENGTHS RATIOS OF ANTIDEPRESSANTS ROUTINELY ANALYSED

Drug	Retention time (min)	Relative retention time	Capacity factor (k')	Wavelength ratio (220/254 nm) (mean ± S.D.; n = 8)
Mix A				
Nordoxepin	3.67	0.41	4.4	3.2 ± 0.04
Desipramine	5.88	0.66	7.6	1.8 ± 0.01
Nortriptyline	7.27	0.81	9.7	2.8 ± 0.05
Cianopramine[a]	8.93	1.00	12	2.0 ± 0.03
Doxepin	10.5	1.18	14	3.1 ± 0.07
Dothiepin	14.6	1.63	20	2.7 ± 0.02
Imipramine	17.9	2.00	25	1.8 ± 0.02
Amitriptyline	22.5	2.52	32	2.6 ± 0.06
Clomipramine	32.1	3.60	46	2.8 ± 0.11
Mix B				
Norfluoxetine	3.61	0.40	4.3	16 ± 0.10
Fluoxetine	6.70	0.75	8.9	17 ± 0.55
Cianopramine[a]	8.98	1.00	12	2.0 ± 0.03
Mianserin	19.8	2.20	28	3.8 ± 0.09
Trimipramine	22.0	2.45	31	1.7 ± 0.05

[a] Internal standard.

linear proportionality between extracted concentration and detector response.

Clean extracts were obtained from post-mortem blood. Cadaveric blood from decomposed cases also showed no interference with the antidepressants although a reduced recovery of extraction, indicated by a lower internal standard peak, was observed in some decomposed specimens. Typical chromatograms of an extracted blood standard and an extract of a post-mortem case blood containing dothiepin at a concentration of 1.0 mg/l, together with dothiepin metabolites are shown in Fig. 2.

The HPLC method described in this paper demonstrates clear advantages over the screening techniques such as radio-immunoassay, enzyme-immunoassay and fluorescence polarization in that it can discriminate parent drugs from metabolites (see Tables II and III), and has good sensitivity from extracted blood samples showing suitability for clinical and forensic work. It also presents an improvement over gas chromatographic methods by providing good separation of all TCA, and other antidepressant drugs. The method provides an advantage over previously reported HPLC methods by allowing quantitation of many commonly used antidepressants in a single extraction. Comparison of absorbance ratios at two wavelengths gives confirmation of peak purity and can enable identification of other eluting compounds. However, the use of absorbance ratio data as an indicator of peak purity is limited to those cases where gross overlap occurs between a compound with a significantly different absorbance ratio from that of the antidepressant concerned. This method, therefore, cannot guarantee the absence of interferences.

The method described was designed to serve the laboratory as a means of confirming the presumptive identification of antidepressants by

TABLE III

LIST OF OTHER ANTIDEPRESSANTS AND METABOLITES, AND OTHER COMPOUNDS EXTRACTED AT A CONCENTRATION OF 1 mg/l

Drug	Retention time (min)	Relative retention time	Capacity factor (k')	Wavelength ratio (220/254 nm)
Other antidepressants and metabolites				
Moclobemide	1.30	0.14	0.9	1.1
Tranylcypromine	1.43	0.15	1.1	27
Dothiepin sulfoxide	1.96	0.22	1.9	3.2
Nomifensine	3.34	0.36	3.9	5.3
Trazodone	3.48	0.38	4.1	2.4
Northiaden	4.98	0.55	6.3	2.8
Protriptyline	5.50	0.60	7.1	7.7
Desmethyl mianserin	5.98	0.65	7.8	4.3
Maprotiline	6.00	0.65	7.8	18
Amoxapine	6.61	0.72	8.7	2.3
Desmethyl clomipramine	10.6	1.16	15	2.8
Other drugs				
Metoclopramide	1.38	0.15	1.0	3.8
Pentobarbitone	1.50	0.16	1.2	8.4
Propranolol	2.15	0.24	2.2	32
Quinine	3.53	0.38	4.2	8.9
Quinidine	3.55	0.39	4.2	9.6
Pheniramine	4.07	0.44	5.0	2.1
Haloperidol	4.80	0.52	6.1	1.4
Pethidine	4.89	0.53	6.2	NR[b]
Norpropoxyphene	5.16	0.56	6.6	NR
Diphenhydramine	7.51	0.82	10	28
Sulforidazine	7.90	0.86	11	1.0
Chlorpheniramine	8.86	0.97	12	5.3
Brompheniramine	10.76	1.17	15	7.8
Methadone	11.16	1.22	15	28
Mesoridazine	12.46	1.36	17	NR
Propoxyphene	12.76	1.39	18	NR
Triprolidine	16.05	1.75	23	1.5
Thiothixene	17.62	1.92	25	1.9
Loxapine	18.6	2.03	26	2.2
Benzhexol	18.96	2.06	27	NR
Promethazine	22.26	2.42	32	0.39
Benztropine	22.75	2.48	32	NR
Cyproheptadine	30.43	3.32	44	2.5
Chlorpromazine	39.19	4.27	57	0.50
Thioridazine	77.76	8.47	113	NR
Dextromethorphan	ND[a]			
Norphetidine	ND			
Phenoxybenzamine	ND			
Prochlorperazine	ND			
Trifluoperazine	ND			

[a] ND = not detected at 1 mg/l.

[b] NR = no ratio (no absorbance at one wavelength: 220 or 254 nm).

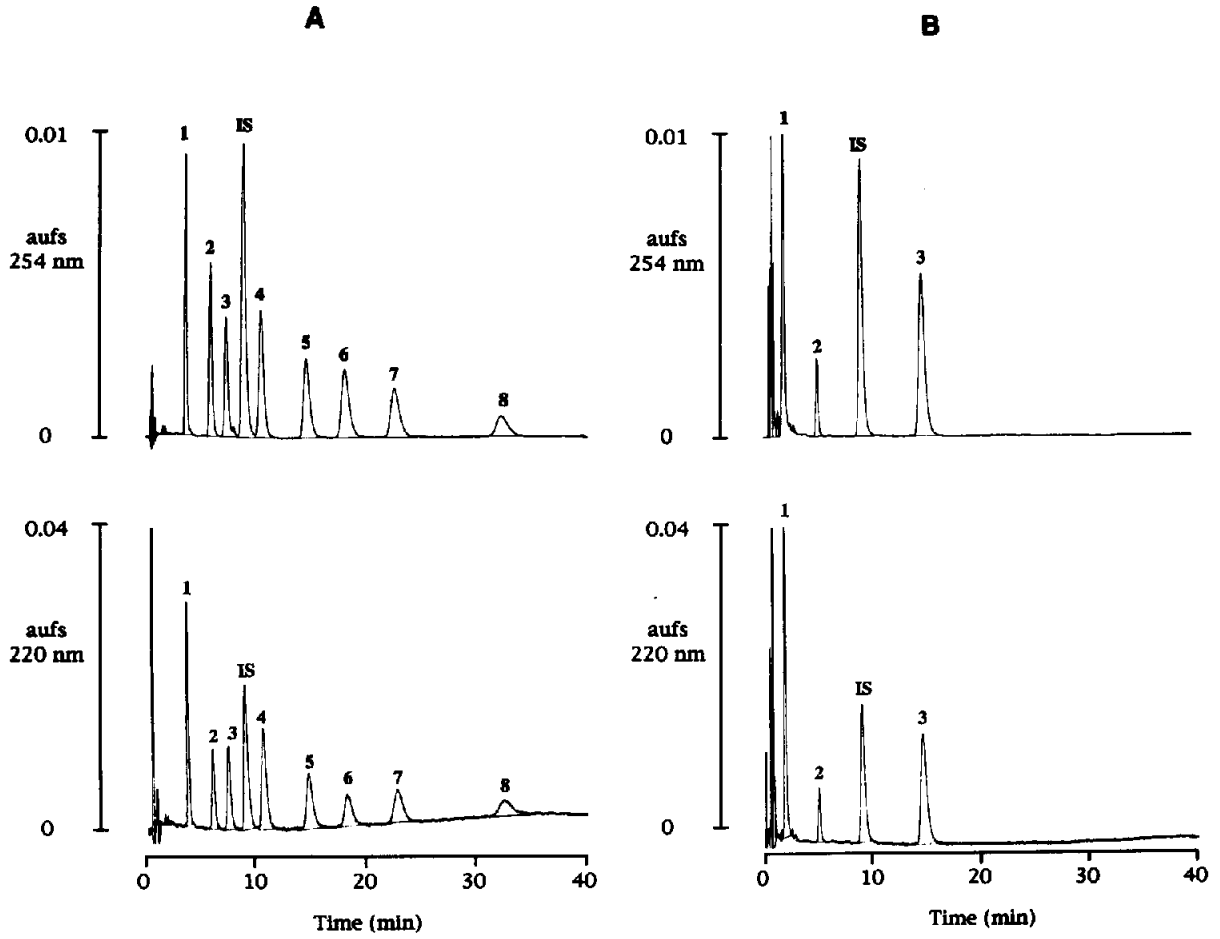

Fig. 2. Chromatograms from analyses at 254 nm in the upper channel, and 220 nm in the lower channel. (A) Extracted blood standard at a concentration of 0.5 mg/l, where: (1) nordoxepin, (2) desipramine, (3) nortriptyline, (4) doxepin, (5) dothiepin, (6) imipramine, (7) amitriptyline, (8) clomipramine, and I.S. = internal standard. (B) Post-mortem case showing dothiepin and metabolites in extracted blood, where: (1) dothiepin sulfoxide, (2) northiaden, (3) dothiepin (1.0 mg/l), and I.S. = internal standard.

capillary gas chromatography with nitrogen phosphorous detection, and then to quantify their presence. The most commonly observed antidepressants in 281 forensic cases analysed over a two year period were dothiepin, amitriptyline, nortriptyline and doxepin (Table V). Of the 137 cases where the concentration was in the toxic range and may have contributed to death, 35 cases involved a single antidepressant substance. The remaining cases involved combinations of antidepressants with other drugs such as benzodiazepines, other antidepressants, antipsychotics, analgesics, alcohol and anticonvulsants, or death resulted from other causes such as hanging, gunshot or drowning. Of these 35 cases, dothiepin was the single drug in 18 cases, doxepin in 9 cases followed by amitriptyline in 4 cases. These data support other studies of antidepressant toxicity in Australia [10], where the rank order of seriousness of overdose index was dothiepin > doxepin > amitriptyline > imipramine > trimipramine > mianserin. Since approximately 12% of all cases for toxicology involve antidepressants, many of which are present in toxicologically sig-

TABLE IV

DETECTION LIMITS AND RECOVERY OF ANTIDEPRESSANTS ROUTINELY ANALYSED

Drug	Blood detection limit (mg/l)	Recovery (%)	
		Blood[b]	Liver[c]
Amitriptyline	0.05	69 ± 6	45
Cianopramine[a]	–	88 ± 7	70
Clomipramine	0.10	57 ± 7	25
Desipramine	0.05	72 ± 6	35
Dothiepin	0.05	79 ± 7	60
Doxepin	0.05	88 ± 6	70
Fluoxetine	0.10	40 ± 4	ND[d]
Imipramine	0.05	78 ± 4	55
Mianserin	0.05	74 ± 7	55
Nordoxepin	0.05	82 ± 6	65
Norfluoxetine	0.10	36 ± 3	ND
Nortriptyline	0.05	67 ± 6	30
Trimipramine	0.05	48 ± 1	25

[a] Internal standard.
[b] Mean ± S.D. (n = 4 determinations).
[c] Single point analysis.
[d] Not determined.

TABLE V

INCIDENCE OF INDIVIDUAL ANTIDEPRESSANTS IN 281 FORENSIC CASES OVER A TWO-YEAR PERIOD (1991–1992)

Drug	Number of cases
Dothiepin	92 (55)[a]
Amitryptyline/Nortriptyline	57 (24)
Doxepin	53 (28)
Imipramine/Desipramine	24 (16)
Mianserin	24 (1)
Trimipramine	13 (9)
Clomipramine	8 (4)
Fluoxetine	8 (0)
Moclobemide	2 (0)
Total	281 (137)

[a] Numbers in parentheses represent the number of cases where the antidepressant concentration was in the toxic range and may have contributed to death.

nificant concentrations, an analytical method capable of identifying and quantifying such compounds is of great importance in the area of forensic toxicology.

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