Application of Gas Chromatography with NPD Detection to the Analysis of Tricyclic Antidepressants in Blood

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Seven tricyclic antidepressants being most frequently used in Poland: imipramine, desipramine, clomipramine, amitriptyline, nortriptyline, doxepin and desmethyldoxepin were examined by means of gas chromatography. An extraction procedure was developed in order to separate these compounds from blood with satisfactory recovery. The chromatographic analysis with NPD detection was performed, which allowed the analytes to be identified in concentrations lower than therapeutic. It was also demonstrated that NPD detector is not very sensitive to the blood matrix constituents. The optimum conditions to provide satisfactory precision of the method in the wide determination range were defined as well.

Siedem najczęściej stosowanych w Polsce trójpierścieniowych leków antydepresyjnych: imipraminę, dezipraminę, klomipraminę, amitryptylinę, nortryptylinę, doksepinę i nordoksepinę, badano metodą chromatografii gazowej. Opracowano proces ekstrakcji tych związków, pozwalający na ich wyodrębnianie z krwi z zadowalającą wydajnością. Pomiary chromatograficzne wykonywano przy użyciu detektora NPD, co stworzyło możliwość identyfikacji badanych analitów w stężeniach mniejszych od stężeń terapeutycznych. Wykazano ponadto, że detektor ten jest mało czuły na obecność składników matrycy biologicznej krwi. Określono również warunki zapewniające uzyskiwanie wyników analitycznych z dostatecznie dużą precyzją w szerokim zakresie oznaczalności.

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At present the most frequently used antidepressants are of tricyclic structure. These are mostly dibenzo[b,f]azepine, dibenzo[a,d]cycloheptadiene and dibenzo[b,e]oxepine derivatives. According to their pharmacological activity they can be distinguished as slightly stimulating (*e.g.* imipramine, clomipramine), strongly stimulating (*e.g.* desipramine, nortriptyline), and sedative drugs (*e.g.* amitriptyline, doxepin) [1,2].

Tricyclic antidepressants are easily absorbed from the alimentary canal and places of parenteral administration. They firmly bind to serum proteins, and especially strongly to tissues (87–96.5%), which causes their concentrations to be considerably greater in tissues than in blood. The antidepressants reach the maximum therapeutic concentration in blood after 2–4 h, which remains steady for 8–24 h, depending on the type of the drug.

The discussed drugs undergo various metabolic transformations, *e.g.* demethylation of side chains, hydroxylation, N-oxidation, and coupling with glucuronic acid. The main metabolites of imipramine, amitriptyline, doxepin, and clomipramine are desipramine, nortriptyline, desmethyldoxepin and desmethylclomipramine, respectively. All these drugs are pharmacologically active and have low therapeutic index. The toxic dose for adults remains within broad range from 500 to 4000 mg. The lethal dose for imipramine, the most toxic compound among the group equals to750 mg, and its concentration in blood plasma exceeding 1500 ng mL^{-1} is considered as very dangerous to human's life, since in blood serum higher than 450 ng mL^{-1} leads to cardiotoxicity [3,4].

Clinical toxicology requires tricyclic antidepressants to be determined mainly with enzymatic immunological methods (group analysis) and also with liquid (HPLC) and gas chromatography (GC). HPLC usually involves ultraviolet detection and provides the detection limit lower than 5 ng mL^{-1} [5,6]. The GC method is coupled with flame ionisation detector (FID) [6], electron capture detector (ECD) [7], nitrogen-phosphorous detector (NPD) [8–10], and mass spectrometrer (MS) [11]. It has been reported [8] that the application of NPD detector allowed one to determine tricyclic antidepressants present at the concentration lower than their therapeutic level.

The aim of this study was to develop a fast and inexpensive GC/NPD procedure for the identification and determination in blood of seven tricyclic antidepressants: imipramine, desipramine, clomipramine, amitriptyline, nortriptyline, doxepin and desmethyldoxepin, which are most frequently used in Poland, in comparison to antidepressants. Therefore, the results of our studies can be of great importance to clinical and forensic toxicology.

EXPERIMENTAL

The procedure of extraction

The studied antidepressants were extracted from blood according to the previously reported procedures [9–13], after some modifications and the following scheme is proposed:

0.1 mL of maproptyline water solution (at a concentration of 400 ng mL^{-1}) is added to 0.2 mL of blood sample. The solution is shaken in a microshaker for 30 s and then 0.1 mL of 4 mol L^{-1} NaOH and 0.8 mL of n-heptane-isopentanol mixture (99:1) are added. The mixture is shaken again in the microshaker for 45 s and then centrifuged (19600 rpm) for 4 min and frozen for 1 h. In the next step, the organic layer required for further analysis is separated and evaporated to dryness at the temperature of 37°C under the stream of nitrogen. The residue is dissolved in 100 µL of methanol.

All reagents used were of an analytical grade and purchased from Merck (Dortmund, Germany).

Maproptyline (commercially known as Ludiomil; not available in Poland) has the similar structure to amitryptiline and doxepin, and in the analytical procedure applied served as the internal standard.

Chromatographic systems

Gas chromatograph, Model FISONS GC 800^{TOP} coupled with NPD 800 detector was used identification and determination of the studied drugs. Capillary column RTX–1 30 m × 0.25 mm (1 µm), coated with 100% dimethylpolisiloxane and megabore column RTX–50 30 m × 0.53 mm (1 µm), coated with 50% phenyl- 50% methylpolisiloxane served for the separation of the analytes, under the air and hydrogen pressure of 65 and 20 kPa, respectively. The sample and detector temperatures were of 260 and 180°C, respectively. The RTX–1 column was operated at the temperature of 250°C with helium as the carrier gas flowing at the rate of 1.2 mL min^{-1} and distributed with the coefficient of 1:10. The temperature of the RTX–50 column was set at 250°C for 2 min, then it was gradually increased (2°C per min) up to 270°C and finally kept at this level for 2 min. Helium was propelled at the rate of 8.0 mL min^{-1} and distributed with the coefficient of 1:5.

RESULTS AND DISCUSSION

The examination of the qualitative analysis conditions

Standard solutions comprising all the examined drugs were used to study the effectiveness of their separation with both columns: RTX–1 and RTX–50. The absolute retention times are given in Table 1. Only five antidepressants could be satisfactory separated when RTX–1 column was employed; yet it did not provide the separation of the following pairs of drugs: imipramine – doxepin and desipramine – desmethyl-doxepin. With the RTX–50 column the separation of six drugs was successively performed, yet nortriptyline and doxepin could not be separated. In order to identify all seven drugs it was necessary to employ both columns, since the overlapping peaks of nortriptyline and doxepin in column RTX–50 are separated in column RTX–1.

Table 1. Retention times of the examined drugs

| Drug	Absolute retention time, s		Retention time with respect to Ludomil
	Column RTX–1	Column RTX–50	Column RTX–50
Amitriptyline	649	308	0.66
Nortriptyline	670	352	0.76
Imipramine	691	334	0.72
Desipramine	722	387	0.84
Clopramine	1075	499	1.08
Doxepine	697	354	0.76
Desmethyldoxepin	715	404	0.66

RTX–50 column was chosen for further studies, since it enables one to identify a greater number of compounds and also allows the analysis to be carried out in the shorter time (less than 10 min). Moreover, it provides the maproptyline (Ludiomil) tetention time of 466 s, thus its peak is markedly separated from the other signals, which is presented in Figure 1.
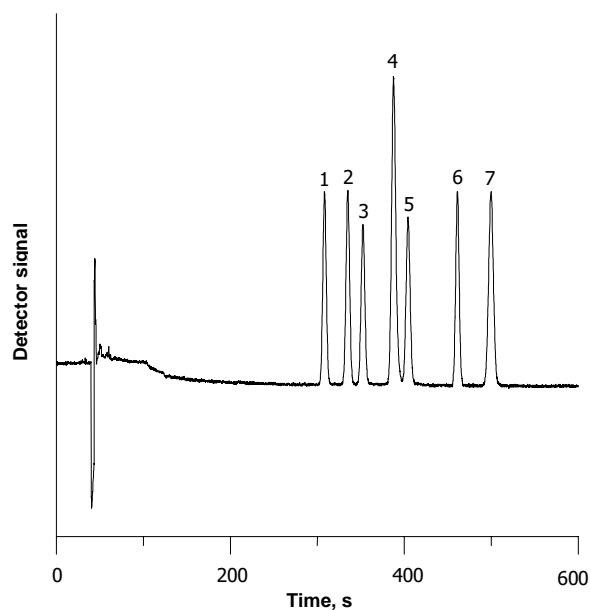
Figure 1. Chromatogram of a mixture of six drugs, 1 – amitriptyline, 2 – imipramine, 3 – nortriptyline, 4 – desipramine, 5 – desmethyldoxepin, 6 – ludiomil (IS), 7 – clomipramine

The retention times of all the analytes studied were evaluated with respect to Ludiomil (Table 1).

Blood extracts free from studied drugs were analysed with RTX–50 column in order to confirm that the blood matrix did not affect the separation of investigated antidepressants, regardless of the blood origin (a living person, a corpse) and its freshness (fresh, decayed). Figure 2 exhibits an exemplary chromatogram of the decayed blood sample taken from a corpse.
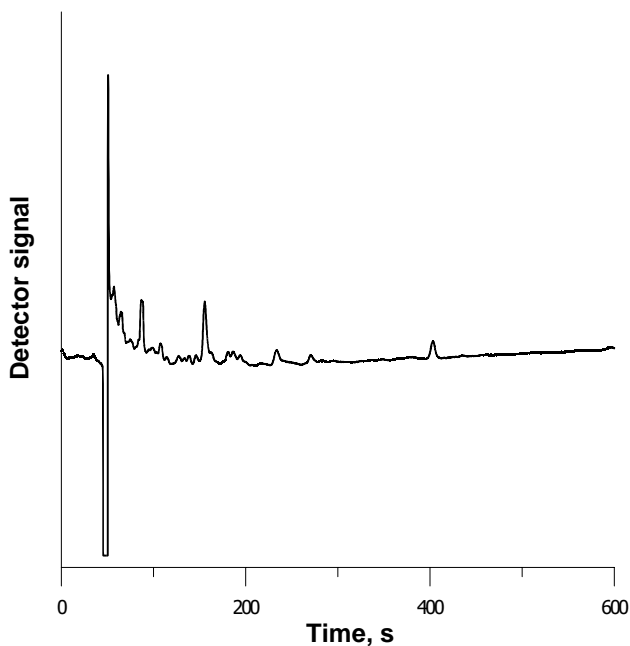
Figure 2. Chromatogram of putrefied blood from a corpse

The examination of the quantitative analysis conditions

The linear ranges of determination of the examined compounds were evaluated with standards containing each drug examined in concentrations ranged (with the internal standard concentration of 100 ng mL^{-1}). Each measurement of the analyte and the internal standard was repeated and the average value was taken for further calculations. Finally, the linear concentration dependencies of the relative chromatographic peak heights were prepared up to concentration values of 1000 ng mL^{-1} for nortriptyline, imipramine, doxepin, desmethyldoxepin, desipramine, and clomipramine, and of 500 ng mL^{-1} for amitriptyline. The respective regression and correlation coefficients are given in Table 2.

Table 2. Regression (a, b) and correlation (r) coefficients of the calibration curves, and detection limits for the examined drugs

Drug	Parameters of the calibration curves			Detection limit, ng mL^{-1}
	a/slope	b/intercept	r/regression coeff.	
Amitriptyline	0.0305	0.0147	0.9937	5.8
Nortriptyline	−0.0764	0.0107	0.9957	15.7
Imipramine	−0.2174	0.0153	0.9921	21.7
Desipramine	−0.1066	0.0171	0.9943	13.2
Clopramine	−0.3258	0.0132	0.9919	22.3
Doxepine	−0.0774	0.0094	0.9980	20.3
Desmethyldoxepin	0.0845	0.0067	0.9980	14.4

In order to determine detection limits of the examined drugs one needed: the peak heights of the internal standards, and the noise signals referring to five different points on a baseline of each drug peak. The respective concentrations were calculated according to the equations in Table 2 and there presented.

Amitriptyline and nortriptyline were used to calculate the extraction recovery. Both compounds were added at various concentrations (100, 200, 300 and 400 ng mL^{-1}) to blood samples free from other drugs and were next extracted according to the procedure described above. The internal standard in concentration of 200 ng mL^{-1} was introduced into the solutions obtained after extraction.

Table 3. Extraction recoveries of amitriptyline and nortriptyline of different concentrations

Drug	Concentration, ng mL^{-1}	Extraction recovery, %
Amitriptyline	100	25.8
	200	43.0
	300	44.4
	400	47.6
Nortriptyline	100	38.6
	200	57.4
	300	65.2
	400	66.6

The signals (relative peak heights) of standard and extracted solutions were compared in order to calculate the extraction recovery, which occurred to be rather constant (*ca* 45% for amitriptyline and 60% for nortriptyline) within the concentration range of 100–400 ng mL^{-1} (Tab. 3).

Blood samples containing known additions of amitriptyline and nortriptyline in concentrations of 75 and 175 ng mL^{-1}, respectively underwent the extraction procedure in order to examine its reproducibility. Amitriptyline and nortriptyline were extracted in the presence of Ludiomil. The measurements were repeated three times for each sample, and the analyses were performed under the same conditions on three different days (Tab. 4). Statistical analysis of the resulting data (analysis of variance in simple classification at the significance level $\alpha = 0.05$) revealed that there was a drift in time observed for the results for nortriptyline, in contrast to stable results for amitriptyline.

Table 4. Reproducibility of analytical signals (relative peak heights) of amitriptyline and nortriptyline within a three-day time (n = 3)

Drug	Concentration, ng mL^{-1}	Day	Signal*	RSD, %
Amitriptyline	75	A	0.679	7.0
		B	0.561	7.8
		C	0.775	3.4
	175	A	1.502	3.5
		B	1.197	8.6
		C	1.596	2.1
Nortriptyline	75	A	0.644	3.2
		B	0.593	2.7
		C	0.576	5.7
	175	A	1.388	5.9
		B	1.309	7.8
		C	1.277	0.6

Finally, amitriptyline and nortriptyline were added to blood samples at various concentrations (50, 100, 150, 200, and 250 ng mL^{-1}), next they were extracted in the presence of Ludiomil, and determined in the mode of relative peak heights. The results were compared with calibration curves for standard solutions and presented in Table 5. It is obvious that determination accuracy for nortriptyline is higher than for

amitriptyline: the determination error for nortriptyline was lower than 5% within the analyte concentration range of 100–200 ng mL^{-1}. However, for both drugs one might risk committing more systematic errors (even of 30–40%). Unfortunately, it may also concern other compounds from the examined group of drugs.

Table 5. Determined amitriptyline and nortriptyline concentrations in blood

Drug	Concentration taken, ng mL^{-1}	Concentration determined, ng mL^{-1}	Relative error of determination, %
Amitriptyline	50	27.6	-44.8
	100	65.9	-34.1
	150	102.8	-31.5
	200	141.8	-29.1
	250	169.4	-32.2
Nortriptyline	50	30.0	-40.0
	100	97.6	-2.4
	150	148.9	-0.7
	200	191.4	-4.3
	250	214.6	-14.2

CONCLUSIONS

The research carried out allows the following evaluation of the proposed analytical method to be carried out: the NPD detector coupled with gas chromatograph has proved its usefulness for identification and determination of tricyclic antidepressants present in blood at concentrations from several to a dozen ng mL^{-1}, which are two orders magnitude lower than FID detector requires. Moreover, the NPD detector exhibits poor sensitivity to matrix effect originating from both: fresh and decayed blood.

Megabore RTX–50 column, capillary RTX–1 column and NPD detector allow reliable identification of seven antidepressants to be carried out under the established separation and detection conditions. Under these conditions the studied drugs can be determined in blood in the wide range of concentrations, even significantly lower than therapeutic.

Extraction of amitriptyline and nortriptyline from blood provides a satisfactory recovery (in the range of 40 to 60%) and good in-a-day reproducibility. However, for a particular blood sample the recoveries did not remain constant within a few day periods and tended to vary to the statistically considerable degree. One should re-

member then that separation of drugs and their determination with gas chromatography method ought to take place directly after extraction of the drugs from blood samples.

Direct application of the standard solutions to the examined drugs after their prior extraction from blood samples does not lead to definite analytical results. Yet, this assumption should be still proved with blood samples comprising a natural composition of the examined compounds (which the authors did not have at their disposal during the research). Therefore, the standard solutions should be extracted in the same way as the analysed samples.

It can be stated that the analytical method developed provides fast and inexpensive identification of very low amounts of the examined tricyclic antidepressant drugs in blood. The quantitative results are promising in terms of their detection limits, precision, and the determination ranges. The accuracy of the method considerably depends on the extraction and calibration conditions. The GC/NPD procedure for reliable determination of all seven antidepressants in blood is still being developed in our laboratories.

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