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Note

Metabolic studies of explosives

IV. Determination of 2,4,6-trinitrotoluene and its metabolites in blood of rabbits by high-performance liquid chromatography—mass spectrometry

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The detection and analysis of 2,4,6-trinitrotoluene (TNT) and its metabolites in body fluids have important applications in the biomedical, environmental and forensic fields [1—6].

Urinary metabolites of TNT in animals have been detected by various groups [3, 7, 8] as well as by this laboratory [5, 6]. The urinary metabolites found in rats [6] include 2-amino-4,6-trinitrotoluene (2-A), 4-amino-2,6-trinitrotoluene (4-A), 2,4-diamino-6-nitrotoluene (2,4-DA) and untransformed TNT It has been assumed [7] that in the metabolism of TNT both reduction and oxidation processes may occur The reduction process possibly involves a stepwise reduction of the nitro group through the intermediate hydroxylamino to the amino group The oxidation process would involve the oxidation of the $-CH_3$ group to form $-CH_2OH$ and $-COOH$ Azoxy compounds could also be formed through oxidation and coupling reactions of the corresponding hydroxylamino-dinitrotoluenes.

As only reduction products were found in the urine of rats fed with TNT [5, 6], we decided to investigate the metabolites of TNT in blood. No information on metabolites of TNT in blood were found in the literature For this research, rabbits were chosen because it was easier to take blood samples from rabbits than from rats High-performance liquid chromatography—mass spectrometry (HPLC—MS) was chosen as the analytical method for the detection and identification of the metabolites since it has already proved to

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be suitable for the analysis of urinary metabolites of TNT [5, 6], because it incorporates good separation and identification characteristics.

EXPERIMENTAL

Equipment

The HPLC—MS system consisted of a high-performance liquid chromatograph interfaced to a magnetic sector mass spectrometer by a variable split-type direct liquid insertion probe interface, and is described in detail elsewhere [9] Positive-ion chemical-ionization (CI) mass spectra were obtained using the mobile phase as CI reagent.

The HPLC columns used were an RP-8 reversed-phase column (10 cm × 4.6 mm I D), particle size 5 μm (Brownlee Labs., Santa Clara, CA, U S A.), with a flow-rate of 1 ml/min, and an RP-8 reversed-phase column (10 cm × 2.0 mm I.D.), particle size 5 μm (Brownlee Labs), with a flow-rate of 70—100 μl/min. Mobile phases were acetonitrile—water at various relative concentrations. UV detector wavelength was 214 nm

Standards, samples and solvents

Standard metabolites were synthesized [5] and analysed by HPLC—MS [5] Purified TNT was obtained from the Israeli Police Analytical Laboratory. Methylene chloride was UV grade (Merck, Darmstadt, F.R G) The HPLC solvents used were UV-grade acetonitrile (Fluka, Buchs, Switzerland) and triple-distilled water The solvents were filtered through a 1 0-μm filter (Whatman, Maidstone, U.K.), and the sample solutions through a 0 5-μm filter (Millipore, Bedford, MA, U.S.A)

Extraction procedure

A 3-ml blood sample (taken from the rabbit's ear) was centrifuged at 12 100 *g* for 5 min at 0—4°C. A 1-ml volume of serum was obtained, which was mixed with 1 ml of water in a 50-ml flask. Methylene chloride (12 ml) was added and stirred vigorously for 30 min. The methylene chloride extract was transferred to a 50-ml centrifuge tube and centrifuged at 27 000 *g* for 5 min at 0—4°C. The aqueous layer formed was discarded with a pasteur pipette. The methylene chloride layer was transferred to a 15-ml glass vial and dried over anhydrous sodium sulphate at 0°C for 1 h The extract was then filtered through a 0 5-μm filter and dried with a flow of nitrogen About 0 1 ml of methylene chloride was added to this residue, the solution transferred to a 1-ml vial and dried again with a flow of nitrogen The residue was then dissolved in acetonitrile and was ready for injection into the HPLC—MS system.

Animal tests

Male rabbits weighing ca 2 kg each were used Single doses of 100 mg of TNT dissolved in 3.5 ml of corn oil were fed with a stomach tube to each of nine rabbits. Two additional rabbits, fed with corn oil only, served as blank reference rabbits. Blood samples (3 ml) were taken from the rabbits 1, 3, 6 and 12 h after feeding

Analysis

Analysis was done by HPLC–MS Samples (1 μl) of the extract solutions were injected into the HPLC–MS system. Quantitative results were obtained by plotting HPLC peak heights versus amount of standard metabolite injected, thus obtaining standard curves that were used for quantitation of sample metabolites.

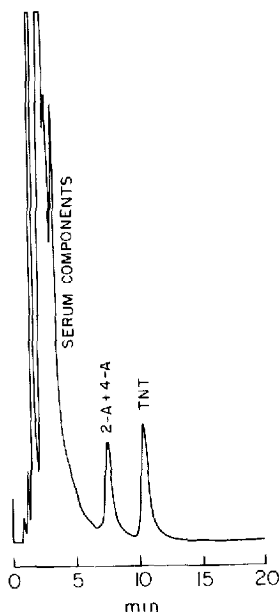
RESULTS AND DISCUSSION

Table I shows the results of the metabolites obtained from the blood of the animal tests. Examples of HPLC profiles (Figs. 1 and 2) and a mass spectrum (Fig 3) are shown Fig 1 shows the HPLC–UV trace of a serum extract from a rabbit fed with TNT Acetonitrile– water (40 60) was used as mobile phase

TABLE I

METABOLITES OF TNT FOUND IN THE BLOOD OF RABBITS

Metabolite	Amount of metabolite found in blood (ng/ml of serum)			
	After 1 h	After 3 h	After 6 h	After 12 h
TNT	66–293	102–306	13–82	4–15
2-A	19–161	41–132	9–47	2–7
4-A	13–96	18–78	6–21	1–8



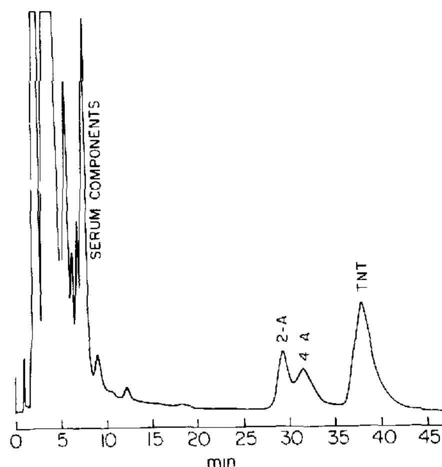


Fig 1 HPLC– UV trace of a serum extract (methylene chloride) from a rabbit fed with TNT Column, RP-8, mobile phase, acetonitrile– water (40 60), flow-rate, 1 ml/min, UV wavelength, 214 nm Amount of 2-A + 4-A, 100 ng, amount of TNT, 175 ng

Fig 2 HPLC–UV trace of a serum extract (methylene chloride) from a rabbit fed with TNT Column, RP-8, mobile phase, acetonitrile–water (25 75), flow-rate, 1 ml/min, UV wavelength, 214 nm Amount of 2-A, 60 ng, amount of 4-A, 55 ng, amount of TNT, 175 ng

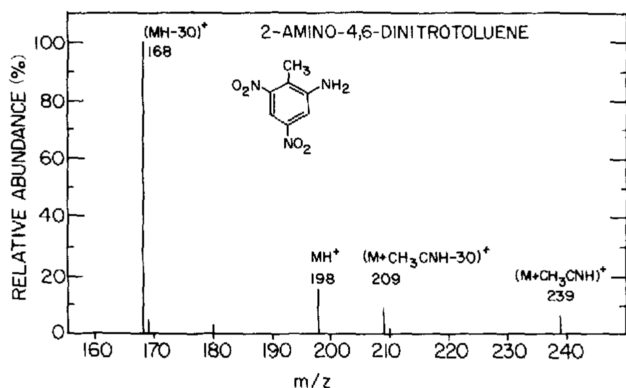


Fig 3 HPLC—MS mass spectrum of 2-A Mobile phase (serving as CI reagent) acetonitrile–water (25 75)

Several large peaks originating from serum components that were not separated during the extraction procedure appear in the chromatogram, but they do not interfere with the metabolites of interest 2-A and 4-A were not separated in this run. In order to separate these isomers, a more polar mixture of acetonitrile—water (25 75) had to be used (Fig 2)

The mass spectrum of 2-A (Fig 3) is a CI mass spectrum using the HPLC mobile phase as CI reagent The peak at m/z 239 is due to the adduct ion $(M + CH_3CNH)^+$, an addition of an acetonitrile ion to the analysed molecule M Loss of 30 mass units from an ion, mainly due to a reduction effect in the ion source [10], is very frequent when water is used as CI reagent In this mass spectrum, two ions are formed in such a way. the base peak ion $(MH - 30)^+$ at m/z 168 and the ion at m/z 209 are due to the loss of 30 mass units from the adduct ion at m/z 239 The ion at m/z 198 is a typical CI MH^+ ion

The CI mass spectrum of 4-A is very similar These isomers can be differentiated only by electron-impact (EI) MS [11]

Results show that the main metabolic pathways of ingested TNT in rabbits, as reflected in the metabolite levels found in their blood, are reduction processes of the nitro group to the amino group, thus forming 2-A and 4-A Comparison of amounts of metabolites found in blood with those found in urine of the same rabbits [12] shows a 100-fold amount in urine This is due to the fact that total amounts of urine (20 ml) were taken for each analysis, while in blood tests only 3-ml samples were taken This is about 1—1 5% of the total amount of blood of these rabbits From these results, it is obvious that urine sampling is preferable for the determination of amounts of TNT metabolites

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