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METABOLIC STUDIES OF EXPLOSIVES

II. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY—MASS SPECTROMETRY OF METABOLITES OF 2,4,6-TRINITROTOLUENE

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SUMMARY

A series of metabolites of 2,4,6-trinitrotoluene were studied by combined high-performance liquid chromatography—mass spectrometry. These metabolites are formed mainly by oxidation and reduction processes. Separations were done on a C_8 reversed-phase column, using acetonitrile—water at various relative concentrations as mobile phases, followed by ultraviolet and on-line mass spectrometry with a direct liquid insertion probe liquid chromatograph—mass spectrometer interface. The mass spectra obtained were chemical-ionization spectra with the mobile phase as reagent. Mass spectra obtained included mainly reduced ions, adduct ions and typical fragment ions. The combination of high-performance liquid chromatographic separation with on-line mass spectrometry was found to be a suitable method for the identification of the investigated metabolites.

INTRODUCTION

The metabolism of explosives in the human body and the analysis of explosives and their metabolites in body fluids are of great importance in several applications.

(1) Biomedical field. Many explosives are toxic [1], therefore inhalation of their vapour presents a major health hazard. Periodical analysis of the body fluids of personnel working in explosives manufacturing plants must be made for the detection of traces of explosives and their metabolites.

(2) Environmental analysis. Improper disposal of obsolete explosives in the environment may cause serious contamination problems [2]. Trace detection of explosives and their metabolites in blood and urine of animals and humans in the disposal area may reveal the extent of contamination.

(3) Forensic application. Trace amounts of explosives absorbed through the pores of a suspect's hands could eventually be detected as metabolites in the blood and urine.

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Two conditions have to be met in order to implement these methods successfully: (1) to have a sensitive analytical method with good separation and identification capabilities, which will enable the analysis and identification of the metabolites in body fluids; (2) to know what metabolites to expect, which means to know the metabolism of explosives in humans and animals.

We have focused our interest on the metabolites of 2,4,6-trinitrotoluene (TNT) because it is one of the most widely used explosives. A few attempts have been made to study the metabolism of TNT in animals [3—7], and based on these results a scheme of the metabolic pathways of TNT was constructed [8]. In the metabolism of TNT both reduction and oxidation processes may occur. The reduction process involves a stepwise reduction of the nitro group through the intermediate hydroxylamino to the amino group. The oxidation process involves the oxidation of the $-CH_3$ group to form $-CH_2OH$ and $-COOH$. Azoxy compounds are also formed through oxidation and coupling reactions of the corresponding hydroxylaminodinitrotoluenes.

High-performance liquid chromatography—mass spectrometry (HPLC—MS) was chosen as the analytical method because it incorporates good separation and identification characteristics. HPLC—MS has proved to be adequate for the analysis of explosives [9].

The purpose of this research was to investigate the use of HPLC—MS as a method for the separation and specification of TNT and its metabolites. Based on the assumed scheme [8] of the metabolism of TNT, we decided to investigate the following metabolites: 2-amino-4,6-dinitrotoluene (2-A), 4-amino-2,6-dinitrotoluene (4-A), 4-hydroxylamino-2,6-dinitrotoluene (4-OHA), 2,4-diamino-6-nitrotoluene (2,4-DA), 2,6-diamino-4-nitrotoluene (2,6-DA), 2,4,6-trinitrobenzoic acid (TNB acid), 2,4,6-trinitrobenzyl alcohol (TNB alcohol), 2,2′,6,6′-tetranitro-4,4′-azoxytoluene (4,4′-Az) and 4,4′,6,6′-tetranitro-2,2′-azoxytoluene (2,2′-Az).

EXPERIMENTAL

Equipment

The HPLC—MS system consisted of a high-performance liquid chromatograph interfaced to a magnetic sector mass spectrometer by a direct liquid insertion probe interface, and is described in detail elsewhere [9].

The column used was an RP-8 reversed-phase column and the mobile phases were acetonitrile—water at various relative concentrations. The flow-rate was 1 ml/min and the UV detector wavelength was 214 nm.

Samples and solvents

The following metabolites were synthesized: 2-A [10], 4-A [11], 2,4-DA [12], 4-OHA [11], 2,2′-Az [13], 4,4′-Az [13], TNB acid [14] and TNB alcohol [15]. Purity was confirmed by thin-layer chromatography, nuclear magnetic resonance spectroscopy and melting point determinations. 2,6-DA was commercially available (Aldrich, Milwaukee, WI, U.S.A.). TNT was obtained in pure form from the Israeli Police Analytical Lab. Metabolites were dissolved in UV-grade acetone (Fluka, Buchs, Switzerland) with the excep-

tion of 2,2′-Az and 4,4′-Az which were dissolved in analytical-grade methylene chloride (Bio-Lab., Jerusalem, Israel). The solvents used were HPLC-grade acetonitrile (Bio-Lab.) and triple-distilled water. The solvents were filtered through a 1.0-μm filter (Whatman, Maidstone, U.K.) and sample solutions were filtered through a 0.5-μm filter (Millipore, Bedford, MA, U.S.A.).

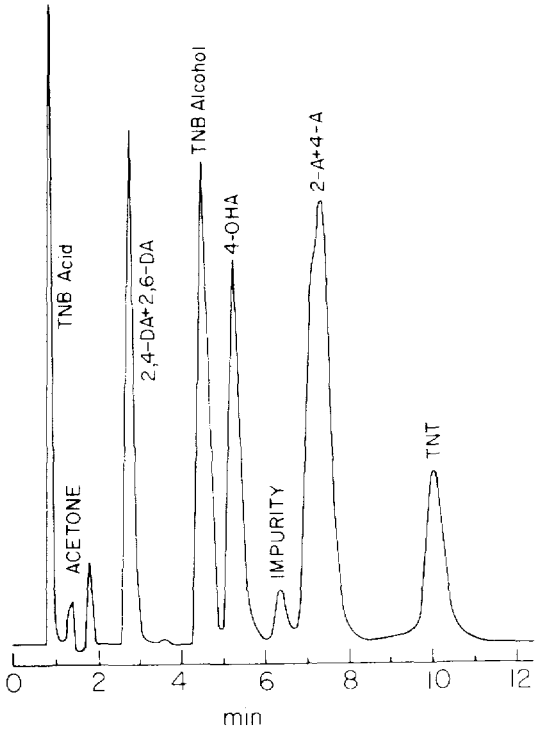

Fig. 1. HPLC—UV trace of a mixture of metabolites containing 2-A, 4-A, 2,4-DA, 2,6-DA, 4-OHA, TNB acid, TNB alcohol and TNT. Column: RP-8; mobile phase: acetonitrile—water (40:60); flow-rate: 1 ml/min; UV wavelength: 214 nm.

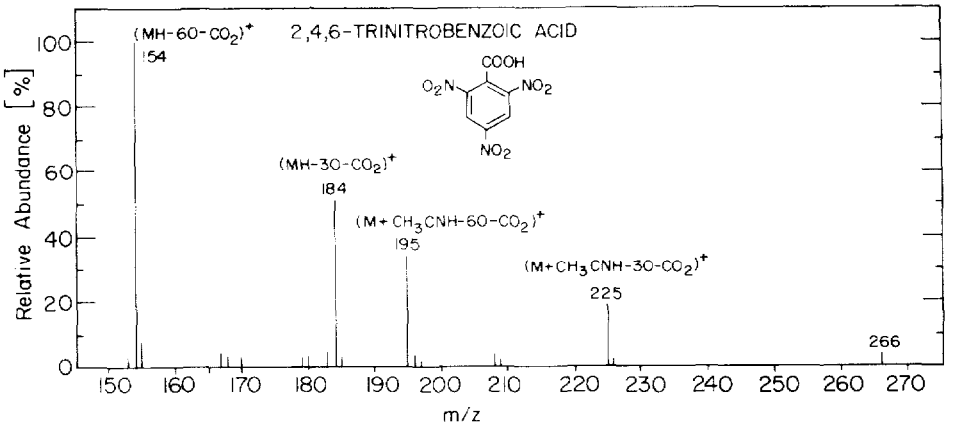

Fig. 2. HPLC—MS mass spectrum of TNB acid. Mobile phase: acetonitrile—water (40:60).

RESULTS AND DISCUSSION

As it is difficult to find one single procedure for the total separation of all the metabolites [16], we used several separate isocratic separations with various mobile phases: acetonitrile—water (20:80) was used to separate TNB acid, 2,6-DA, 2,4-DA and TNB alcohol; acetonitrile—water (28:72) was used to separate TNB acid, TNB alcohol, 4-OHA, 2-A, 4-A and TNT; acetonitrile—water (40:60) was used to separate TNB acid, TNB alcohol, 4-OHA and TNT; and acetonitrile—water (60:40) was used to separate TNT, 2,2′-Az and 4,4′-Az.

Acetonitrile—water (40:60) was used as mobile phase for the separation of a mixture of metabolites which included 2-A, 4-A, 2,4-DA, 2,6-DA, 4-OHA, TNB acid, TNB alcohol and TNT (Fig. 1). 2-A and 4-A as well as 2,4-DA and 2,6-DA were not separated. The HPLC solvent serves as a chemical-ioniza-

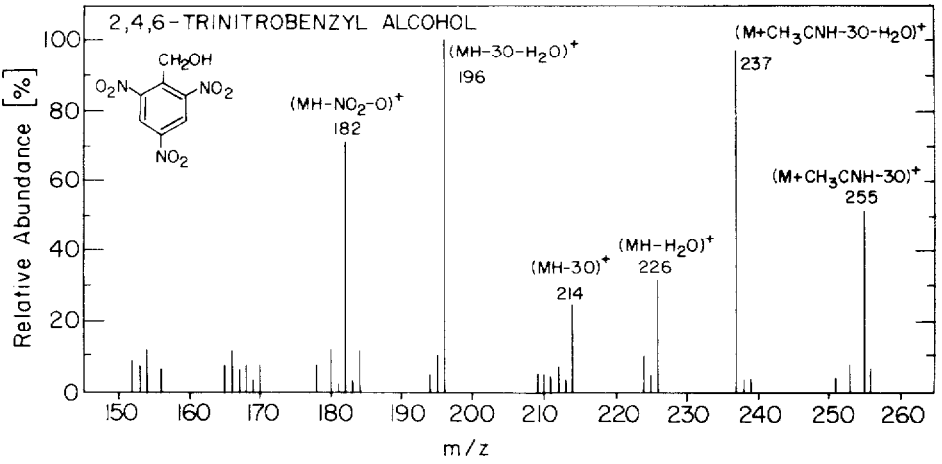

Fig. 3. HPLC—MS mass spectrum of TNB alcohol. Mobile phase: acetonitrile—water (40:60).

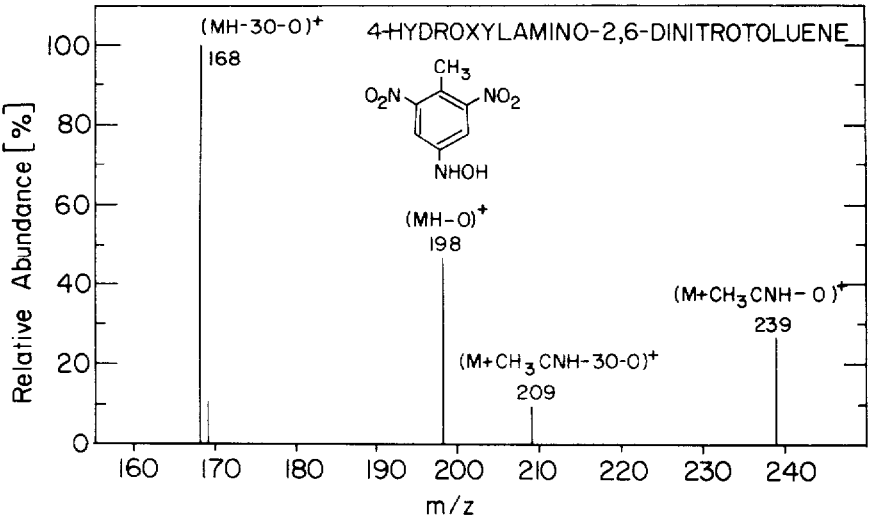

Fig. 4. HPLC—MS mass spectrum of 4-OHA. Mobile phase: acetonitrile—water (40:60).

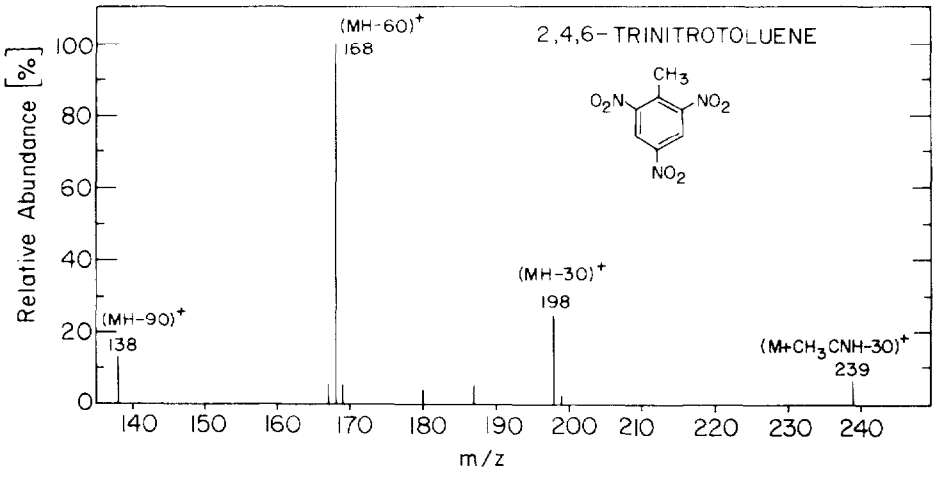

Fig. 5. HPLC—MS mass spectrum of TNT. Mobile phase: acetonitrile—water (40:60).

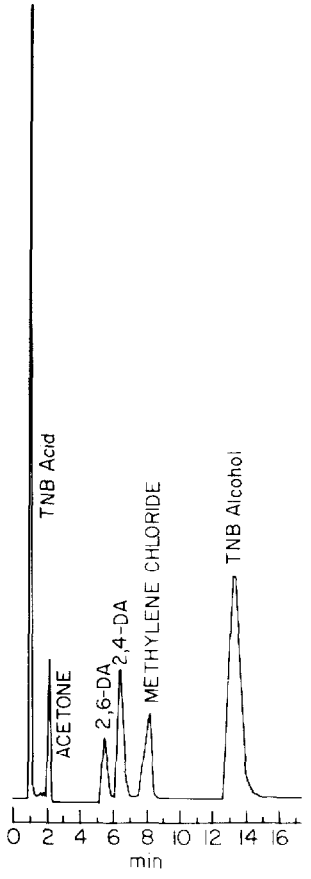

Fig. 6. HPLC—UV trace of a mixture of metabolites containing TNB acid, 2,4-DA, 2,6-DA and TNB alcohol. Column: RP-8; mobile phase: acetonitrile—water (20:80); flow-rate: 1 ml/min; UV wavelength: 214 nm.

tion reagent [9]. The HPLC—MS mass spectrum of TNB acid (Fig. 2) is characterized by fragment ions which are due to additive loss of CO_2 and 30 or 60 mass units from the MH^+ ion and from the adduct ion $(M + CH_3CNH)^+$. This loss of 30 mass units is mainly due to a reduction process of the nitro compound to the corresponding amine in the chemical-ionization source in the presence of water [17]. Loss of 60 mass units corresponds to the reduction of two nitro groups.

The HPLC—MS mass spectrum of TNB alcohol (Fig. 3) is characterized by ions formed by reduction of a nitro group to an amine which is in part followed by loss of H_2O: the reduced adduct ion at *m/z* 255 $(M + CH_3CNH - 30)^+$ and its fragment ion at *m/z* 237 $(M + CH_3CNH - 30 - H_2O)^+$ and the reduced protonated molecular ion at *m/z* 214 $(MH - 30)^+$ and its fragment ion $(MH - 30 - H_2O)^+$ at *m/z* 196.

The HPLC—MS mass spectrum of 4-OHA (Fig. 4) is characterized by a set of fragment ions formed by the loss of O: at *m/z* 239 $(M + CH_3CNH - O)^+$, at *m/z* 209 $(M + CH_3CNH - 30 - O)^+$, at *m/z* 198 $(MH - O)^+$ and at *m/z* 168 $(MH - 30 - O)^+$. The HPLC—MS mass spectrum of TNT (Fig. 5) has a base peak at *m/z* 168 due to an ion formed by the reduction of two nitro groups. The spectrum shows also an ion at *m/z* 138 formed probably by the reduction of three nitro groups. This ion does not appear when the percentage of water in the solvent is smaller.

Acetonitrile—water (20:80) was used as mobile phase for the separation of a mixture of metabolites which included TNB acid, 2,4-DA, 2,6-DA and TNB alcohol (Fig. 6). The retention times of TNT, 2,2′-Az and 4,4′-Az (which were also included in this mixture) were above 30 min; these compounds were therefore separated — for practical considerations — by another solvent mixture. The methylene chloride peak originates from the solution of azoxy compounds.

The HPLC—MS mass spectra of TNB acid and TNB alcohol are similar to

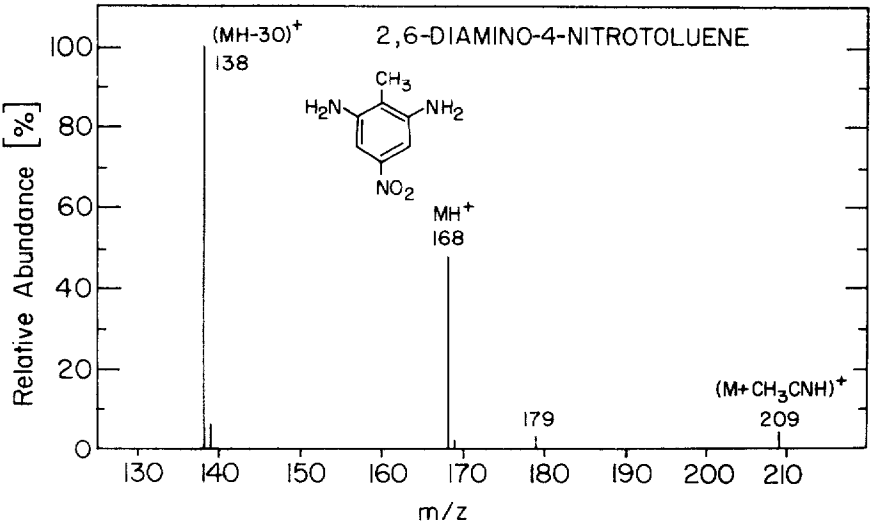

Fig. 7. HPLC—MS mass spectrum of 2,6-DA. Mobile phase: acetonitrile—water (20:80).

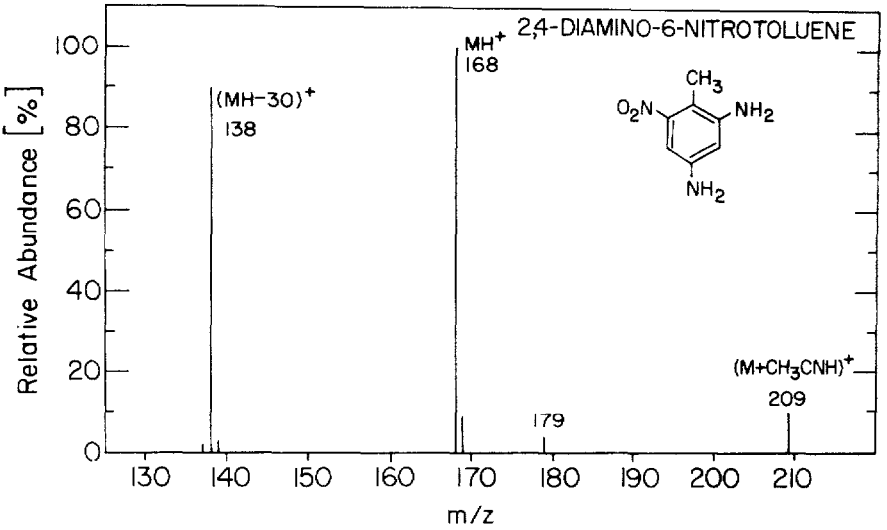

Fig. 8. HPLC—MS mass spectrum of 2,4-DA. Mobile phase: acetonitrile—water (20:80).

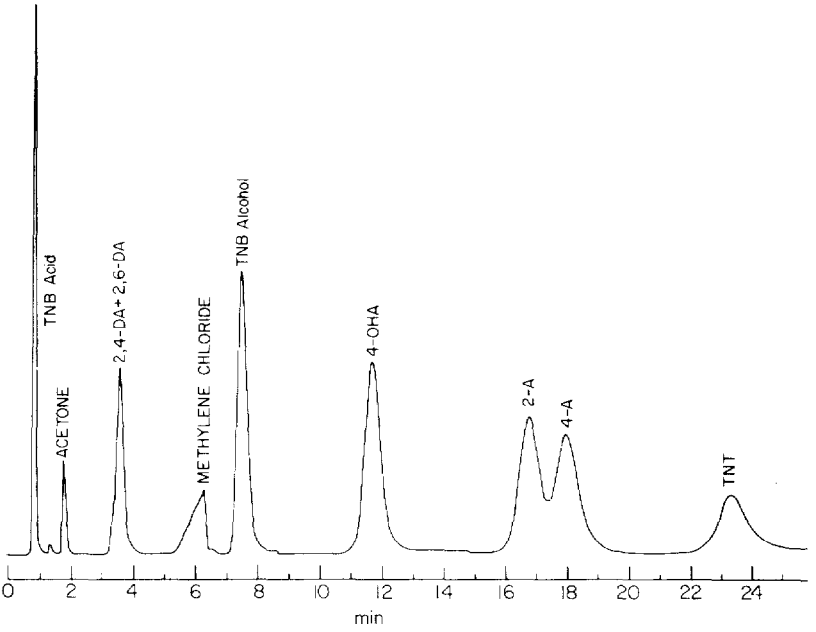

Fig. 9. HPLC—UV trace of a mixture of metabolites containing TNB acid, 2,4-DA, 2,6-DA, TNB alcohol, 4-OHA, 2-A, 4-A and TNT. Column: RP-8; mobile phase: acetonitrile—water (28:72); flow-rate: 1 ml/min; UV wavelength: 214 nm.

those discussed previously, with small differences in the relative peak abundances. The HPLC—MS mass spectra of the two isomers 2,6-DA (Fig. 7) and 2,4-DA (Fig. 8) contain the same ions: MH^+ at *m/z* 168, $(MH - 30)^+$ at *m/z* 138 and a small adduct ion $(M + CH_3CNH)^+$ at *m/z* 209. The relative abundances of the two ions MH^+ and $(MH - 30)^+$ are different in both isomers, but we cannot attribute these differences to their characterization. Differentia-

tion between these two isomers by mass spectrometry is possible only by electron-impact ionization [8].

Acetonitrile—water (28:72) was used as mobile phase for the separation of a mixture of metabolites which included TNB acid, 2,4-DA, 2,6-DA, TNB alcohol, 4-OHA, 2-A, 4-A and TNT (Fig. 9). 2,4-DA and 2,6-DA were not separated. The purpose of this chromatographic run was to separate, even without baseline, the two isomers 2-A and 4-A. TNT appears at a large retention time and the azoxy compounds, which were also part of the mixture, had a retention time above 30 min.

The HPLC—MS mass spectra of TNB acid, TNB alcohol, 4-OHA and TNT are similar to those discussed previously, with small differences in the relative peak abundances. The HPLC—MS mass spectra of the two isomers 2-A

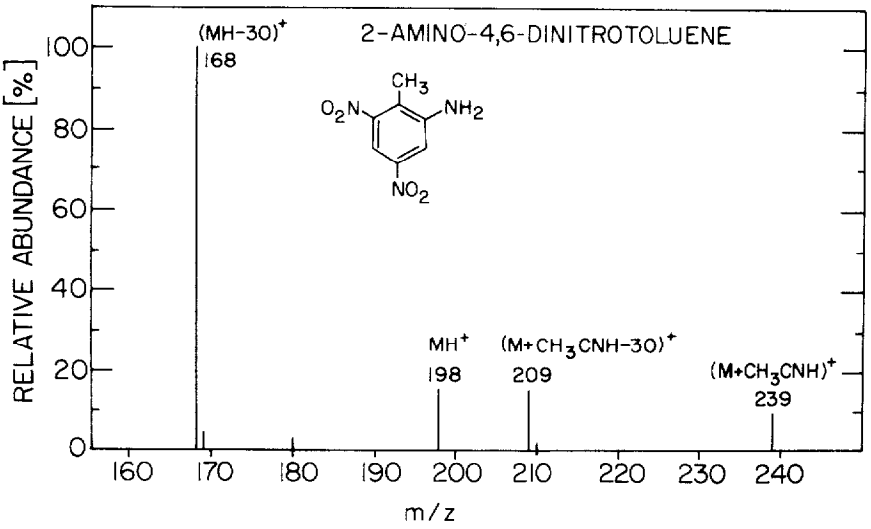

Fig. 10. HPLC—MS mass spectrum of 2-A. Mobile phase: acetonitrile—water (28:72).

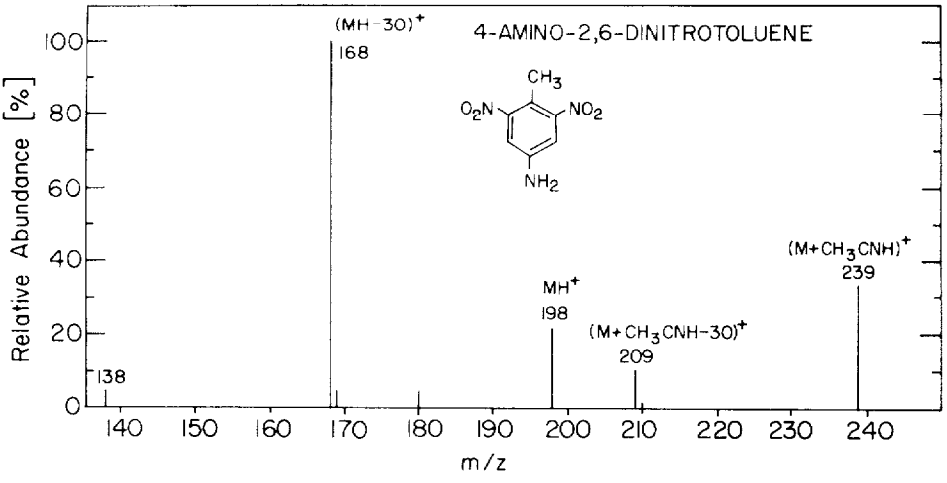

Fig. 11. HPLC—MS mass spectrum of 4-A. Mobile phase: acetonitrile—water (28:72).

(Fig. 10) and 4-A (Fig. 11) contain the same ions: a base peak at *m/z* 168 due to the $(MH - 30)^+$ ion, an MH^+ ion at *m/z* 198 and the adduct ions $(M + CH_3CNH)^+$ at *m/z* 239 and $(M + CH_3CNH - 30)^+$ at *m/z* 209. Differentiation between these two isomers by mass spectrometry is possible only by electron-impact ionization [8].

Acetonitrile—water (60:40) was used as mobile phase for the separation of a mixture of metabolites which included 2,2′-Az, 4,4′-Az and TNT (Fig. 12).

The HPLC—MS mass spectrum of TNT is similar to the one discussed previously, with differences in the relative peak abundances. The HPLC—MS mass spectra of the two isomers 2,2′-Az (Fig. 13) and 4,4′-Az (Fig. 14) contain

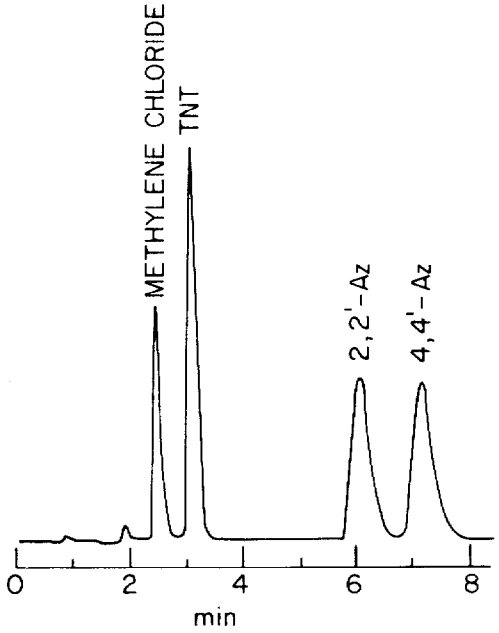

Fig. 12. HPLC—UV trace of a mixture of metabolites containing 2,2′-Az, 4,4′-Az and TNT. Column: RP-8; mobile phase: acetonitrile—water (60:40); flow-rate: 1 ml/min; UV wavelength: 214 nm.

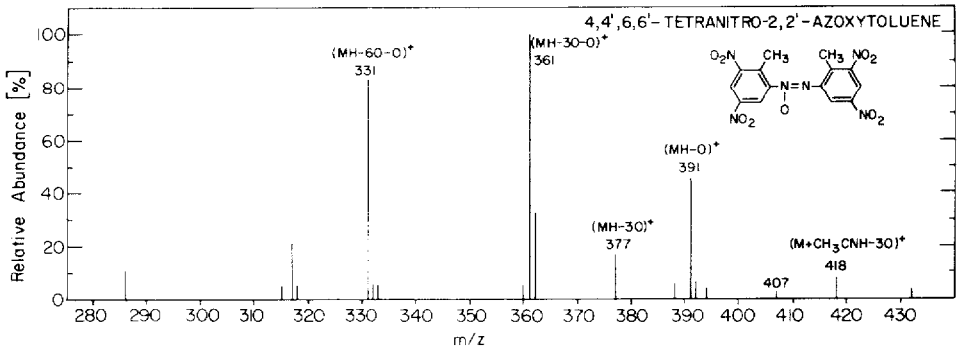

Fig. 13. HPLC—MS mass spectrum of 2,2′-Az. Mobile phase: acetonitrile—water (60:40).

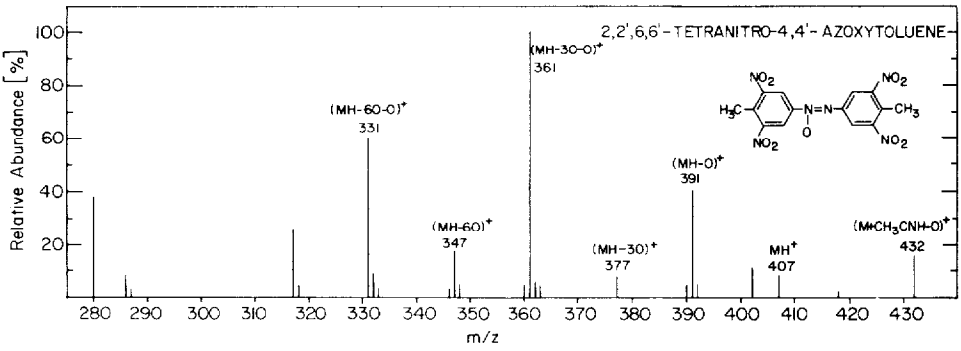

Fig. 14. HPLC—MS mass spectrum of 4,4'-Az. Mobile phase: acetonitrile—water (60:40).

the same ions: $(MH - 30 - O)^+$ at *m/z* 361 (base peak), $(MH - 60 - O)^+$ at *m/z* 331, $(MH - 30)^+$ at *m/z* 377, $(MH - O)^+$ at *m/z* 391, MH^+ at *m/z* 407, $(M + CH_3CNH - 30)^+$ at *m/z* 418 and $(M + CH_3CNH - O)^+$ at *m/z* 432. Differentiation between these two isomers by mass spectrometry was possible only by electron-impact ionization [8].

The use of this HPLC—MS method for the analysis of metabolites of TNT has been demonstrated by an experiment with laboratory animals. Single doses of TNT were fed to male Sprague—Dawley rats by means of a stomach tube. These doses contained 20 mg of TNT dissolved in 1 ml of corn oil. Urine was collected after 12, 24 and 48 h. TNT and its metabolites were extracted from the urine with toluene. HPLC—MS analysis of these extracts showed amounts between 1 and 20 μg of TNT, 2-A and 4-A, and amounts of up to 200 ng of 2,4-DA.

CONCLUSIONS

Separation and identification of the metabolites of TNT were made possible by a combination of several isocratic HPLC separations followed by on-line mass spectrometric identification. Pairs of isomers which could not be differentiated by MS were separated by HPLC. The combination of several short isocratic HPLC runs was found to be preferable to a very long gradient run.

The sensitivity of the HPLC—MS system was found to be between 100 ng and 1 μg per μl injected, while the sensitivity of the HPLC system alone (with UV detection) was 1—10 ng per μl injected. The reduced sensitivity of the HPLC—MS system versus the HPLC with UV detection is caused by the low splitting ratio of the HPLC—MS interface.

The use of microbore columns is planned which will allow a smaller flow-rate. This will increase the splitting ratio of the interface, thus increasing the sensitivity by one to two orders of magnitude.

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