both cold and isolation stress increased the heart's sensitivity to isoproterenol and speculated that steroid hormones released by stress may have sensitized the myocardium to the β-agonist. Raab (15) also suggested that stress-induced discharge of adrenal corticoids may enhance the cardiotoxicity of endogenously released catecholamines. In discussing unexpected sudden death during sleep, he implicated a combination of 17-hydroxycorticosteroids, which reach peak levels during sleep, and dream-induced catecholamine discharges as being contributory to a fatal cardiotoxic interaction (16). The prolonged administration of steroids followed by exposure to catecholamines, as seen in the present study, may mimic the hormonal milieu produced by chronic stress, resulting in the similarity of toxic sequelae.

The applicability of these experimental findings to the clinical setting has to be determined. However, the data point out the potential danger of chronic steroid treatment. In such instances, there might be increased risk not only from administered catecholamines but also from agents that potentiate the effects of endogenous catecholamines, such as aminophylline and possibly monoamine oxidase inhibitors, and from agents that produce catecholamine discharge, such as tyramine, an ingredient of many foods.

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Application of High-Pressure Liquid Chromatography and Thermal Energy Analyzer to Analysis of Trinitroglycerin and Its Metabolites in Blood

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Abstract □ A highly selective and sensitive analytical procedure for the determination of trinitroglycerin and four metabolites in whole blood was developed. Trinitroglycerin and its metabolites were extracted from whole blood with ethyl acetate and analyzed by high-pressure liquid chromatography using the thermal energy analyzer detector. Linearity of response was observed over the 1–1000-ng range. The applicability of this method to the analysis of whole blood from dogs orally dosed with trinitroglycerin is described.

Keyphrases □ Trinitroglycerin—high-pressure liquid chromatographic analysis of parent drug and metabolites in whole blood □ High-pressure liquid chromatography—analysis of trinitroglycerin and metabolites in whole blood □ Thermal energy analyzer—analysis of trinitroglycerin and metabolites in whole blood

The analysis of trinitroglycerin in blood has been performed primarily by electron-capture GLC (1–3). Although this method possesses high sensitivity and some selectivity for trinitroglycerin, it requires extensive cleanup procedures and solvent purification. Furthermore, it does not adequately account for the primary metabolites of trinitroglycerin, the isomeric mono- and dinitroglyceryl esters. A recent report (4) described the detection of ethylene glycol dinitrate esters in water by use of the thermal energy analyzer. It was thought that the analyzer detection system also may be applicable to the analysis of trinitroglycerin in complex matrixes such as whole blood.

This report describes the development of a high-pressure liquid chromatographic (HPLC) method to determine trinitroglycerin as well as the primary isomeric dinitro- and mononitroglyceryl metabolites in whole blood. The application of this methodology to the blood of dogs orally dosed with trinitroglycerin is described.

EXPERIMENTAL

Reagents—Acetone[1] and ethyl acetate[1] were glass distilled. Trinitroglycerin[2] was used as a powder (10% trinitroglycerin in lactose). *N*-Nitrosodipropylamine[3] was supplied as a standard solution in isooctane. All other chemicals and solvents were standard reagent grade and were used without further purification. Authentic samples of 1,3- and 1,2-dinitroglycerols and 1- and 2-mononitroglycerols were obtained[4].

Apparatus—The chromatographic system consisted of a liquid

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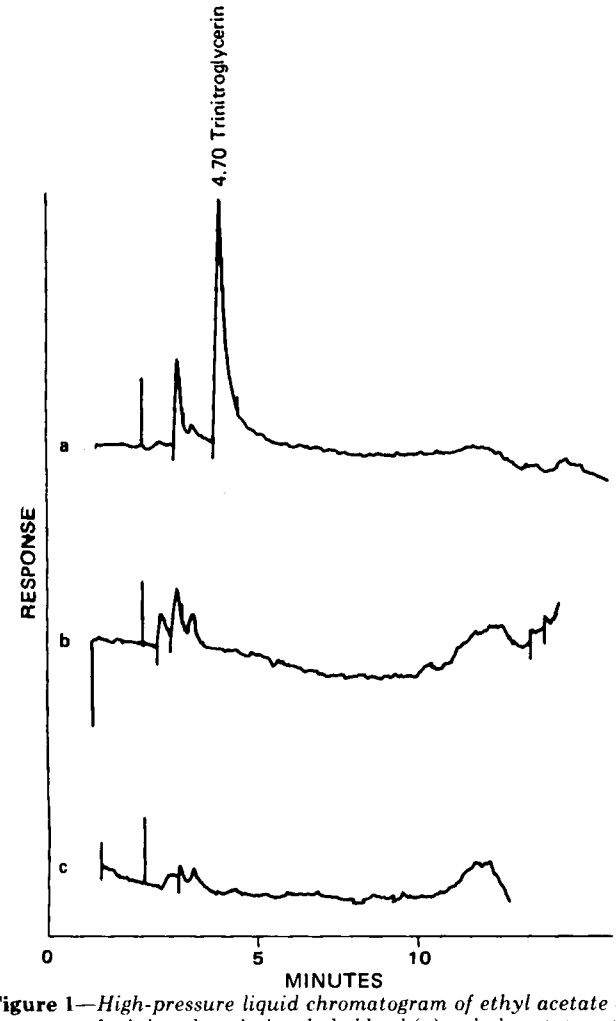

Figure 1—*High-pressure liquid chromatogram of ethyl acetate concentrates of trinitroglycerin in whole blood (a), ethyl acetate extract of whole blood (b), and ethyl acetate concentrate (c).*

chromatograph[5] with gradient elution capability. A 250 × 4.6-mm Lichrosorb Si-60[6] (5-μm) column was used. The solvent system was isooctane–ethyl acetate–acetone, 54:6:40 (v/v) for 10 min isocratically and programmed to 9:1:90 (v/v) over 5 min at a flow rate of 1.4 ml/min. The detection of trinitroglycerin was accomplished with a thermal energy analyzer[7] using the following parameters: furnace temperature, 450°; argon flow rate, 15 ml/min; and oxygen flow rate, 5 ml/min.

Extraction—Whole blood, 2 ml, was combined with 100 μl of 1 *M* $AgNO_3$. The silver nitrate was added to inhibit trinitroglycerin degradation before extraction (3). Extraction was achieved by rapid injection of ethyl acetate (2 ml, then 5 × 1 ml) into the whole blood solution. The organic layers were drawn off after each injection, combined, dried, and filtered through a short column of anhydrous sodium sulfate. The dried extract was concentrated to 500 μl using a gentle nitrogen stream. Then 500 μl of isooctane was added, and the solution was concentrated to 500 μl.

Dosing—A female beagle[8] dog (13.5 kg) was fasted overnight, with free access to water, and dosed by gavage over 10 min with 6.7 g of lactose containing 10% trinitroglycerin (670 mg) incorporated into canned dog food. At various times thereafter, blood (2 ml) was drawn from the cephalic vein into a heparin-containing syringe and then was placed in a vial containing silver nitrate.

Quantitation—Quantitation of the samples was achieved by the internal standard method; the internal standard was *N*-nitrosodipropylamine. A known amount of the internal standard was added to the sample before extraction. Peak areas were determined with the aid of an integrator–recorder[9].

[5] Model 3500B, Spectra-Physics, Santa Clara, Calif.
[6] E. Merck, Darmstadt, West Germany.
[7] Model 502/LC, Analytical Instrument Division, Thermo Electron Corp., Waltham, Mass.
[8] AKC registered, Marshall Research Animals, North Rose, N.Y.
[9] Hewlett-Packard model 3380A.

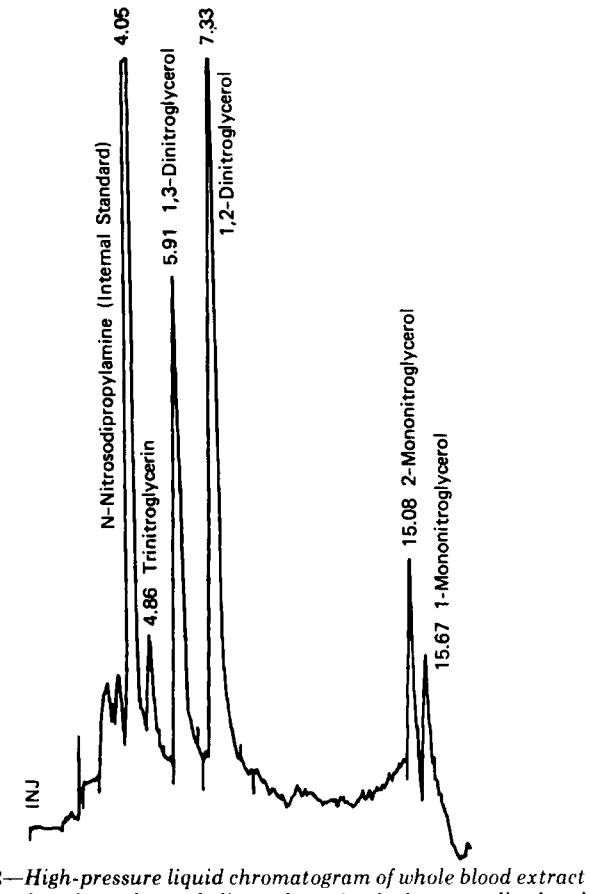

Figure 2—*High-pressure liquid chromatogram of whole blood extract of trinitroglycerin and metabolites after 10 min in an orally dosed dog.*

RESULTS AND DISCUSSION

HPLC was applied previously to the analysis of trinitroglycerin and the primary hydrolysis products, 1,3- and 1,2-dinitroglycerols, in wastewaters from trinitroglycerin production (5). The sensitivity and specificity of this technique were improved greatly when it was coupled to the thermal energy analyzer detector.

Figure 1 shows a normal-phase HPLC profile of an ethyl acetate extract of trinitroglycerin spiked in whole blood, an unspiked ethyl acetate extract of whole blood, and an ethyl acetate solvent control concentrate using the thermal energy analyzer detector. The relatively interference-free baseline of the spiked and unspiked whole blood extracts

Table I—Relative Responses and Detection Limits for Trinitroglycerin and Metabolites

Compound	Response Ratio, DPN[a]/compound	Lower Limit of Detection[b], ng
Trinitroglycerin	0.89	0.5
1,3-Dinitroglycerol	0.90	1.0
1,2-Dinitroglycerol	1.4	1.0
2-Mononitroglycerol	2.1	3.0
1-Mononitroglycerol	2.6	3.0

[a] *N*-Nitrosodipropylamine. [b] Per 100-μl injection.

Table II—Precision of the HPLC–Thermal Energy Analyzer Method for Trinitroglycerin [a]

Spiked	Observed (Mean ± *SD*)	Range[b]
13.2	12.5 ± 1.5	10.5–14.6
37.6	34.7 ± 2.0	32.2–37.0
75.2	73.1 ± 6.7	65.1–82.1

[a] Nanograms per milliliter of whole blood. [b] $n = 5$.

Table III—Quantitative Distribution of Trinitroglycerin and Metabolites (Micrograms per Milliliter) in Dog Blood after Oral Administration of Trinitroglycerin

Compound	Minutes										
	10	20	30	60	90	120	150	180	240	300	360
Trinitroglycerin	0.019	—	—	—	—	—	—	—	—	—	—
1,3-Dinitroglycerol	0.081	0.107	0.100	0.161	0.285	0.723	0.458	1.50	0.545	0.628	0.192
1,2-Dinitroglycerol	0.278	0.308	0.293	0.425	0.775	1.73	0.915	3.89	1.20	1.13	0.315
1-Mononitroglycerol	0.085	0.094	0.131	0.226	0.308	0.749	0.566	0.758	1.17	2.25	0.707
2-Mononitroglycerol	0.058	0.061	0.099	0.225	0.310	0.748	0.676	0.955	1.42	3.37	0.907

demonstrates the selectivity of the thermal energy analyzer detector for trinitroglycerin in the presence of numerous coextractants. Ethyl acetate was chosen as the extraction solvent because of its ability to extract the more polar mono- and dinitroglyceryl metabolites from whole blood; these metabolites also are responsive to the thermal energy analyzer detector. This selectivity for organonitrates permits the quantitative analysis of the 1,2- and 1,3-dinitroglyceryl and 1- and 2-mononitroglyceryl esters (Fig. 2), which could only be qualitatively estimated by previous GLC methods (2, 3). Linear gradient programming allows for the analysis of trinitroglycerin and its four nitro-containing metabolites in 17 min. This programming also flushes the column to remove nonresponsive polar materials and it prolongs column life.

Internal as well as external standard quantitation methods can be applied to the quantitative analysis of trinitroglycerin using the thermal energy analyzer. With *N*-nitrosodipropylamine as an internal standard, the relative responses of trinitroglycerin and its metabolites to this standard, along with the lower limits of detection for each compound, appear in Table I. For trinitroglycerin, a linear response was obtained over the 1–1000-ng range with a correlation coefficient of 0.99.

Ethyl acetate proved to be an efficient extraction solvent for trinitroglycerin and its metabolites in whole blood. Since ethyl acetate readily emulsifies with whole blood upon shaking, rapid injection of the ethyl acetate into the blood from a syringe and removal of the separated phases by syringe provided adequate extraction efficiency with little emulsion problem. Due to the partitioning of this solvent in whole blood, repeated injections were performed for quantitative removal. The precision of the HPLC–thermal energy analyzer method is shown in Table II, which gives recovery values for spiked blood samples.

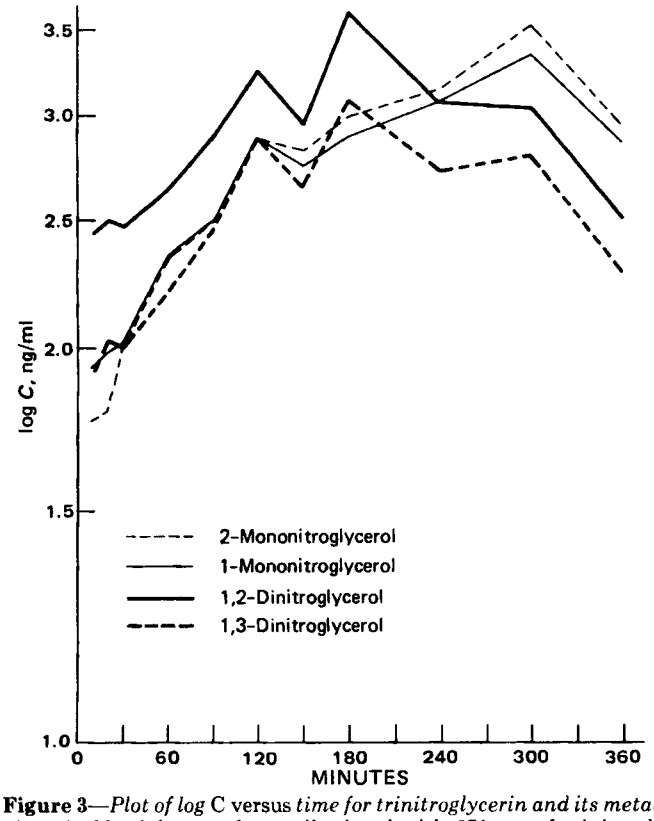

Figure 3—*Plot of log* C versus *time for trinitroglycerin and its metabolites in blood from a dog orally dosed with 670 mg of trinitroglycerin.*

The sensitivity of this method for whole blood was 0.5 ng (25% scale deflection) with the thermal energy analyzer detector at an attenuation of 8. The sensitivity of previously reported GLC methods using electron-capture detection was 0.5–0.1 ng in serum only after extensive cleanup and sample preparation (2, 3). The present method has the advantage of minimal sample preparation and is useful in situations where large samples of serum are not readily available (such as rat and mouse studies); it should be applicable to the study of therapeutic doses of trinitroglycerin in humans.

To test the applicability of the method, a beagle dog was dosed orally (force fed) with trinitroglycerin (49.6 mg/kg). Blood samples were removed at various times and analyzed over 6 hr. The quantitative distribution of trinitroglycerin and its metabolites appears in Table III. As reported previously (3, 6), trinitroglycerin is metabolized rapidly; no trinitroglycerin (<0.5 ng/ml) was detected 20 min after administration. However, the 1,2- and 1,3-dinitroglyceryl and the 1- and 2-mononitroglyceryl metabolites increased to high levels in a parallel fashion and started to decline 5 hr after administration. This finding is consistent with results reported by Hodgson and Lee (7), who found maximum carbon-14 activity in blood 4 hr after oral administration of [^{14}C]trinitroglycerin to rats. A plot of log *C versus* time appears in Fig. 3.

The relative distribution of metabolites in the blood observed in this study paralleled that found by DiCarlo *et al.* (8) in the urine of rats orally dosed with [^{14}C]trinitroglycerin, indicating similar disposition routes of trinitroglycerin for the two species. In both cases, the deesterification appeared to be stepwise.

The data show that there was a slight preference to deesterify at the primary carbon atom. In the early stages of metabolism, the 1,2-dinitroglycerol concentration was three times that of the 1,3-dinitroglycerol isomer. This ratio fell to <2.0 (the expected value if deesterification occurred equally at primary and secondary carbons) at 6 hr. At the same time, the ratio of 2-mononitroglycerol to 1-mononitroglycerol increased from an initial value of 0.68 to 1.5 over 5 hr. Since the source of 2-mononitroglycerol is 1,2-dinitroglycerol (assuming a stepwise process) and the source of 1-mononitroglycerol is both the 1,2- and 1,3-dinitroglycerols, the ratio increase of 2-mononitroglycerol to 1-mononitroglycerol indicates that the 2-position is less reactive to deesterification than is the 1-position.

The present study demonstrates the utility of the thermal energy analyzer detector in the analysis of organonitrates in blood. The application of this method to tissues and other complex matrixes should be useful in pharmacological studies.

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