Determination of Nitroglycerin and Its Dinitrate Metabolites in Human Plasma by High-Performance Liquid Chromatography with Thermal Energy Analyzer Detection

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Abstract □ A highly selective and sensitive high-performance liquid chromatographic assay employing a thermal energy analyzer as the detector for nitroglycerin and its dinitrate metabolites in human plasma has been developed. Prior to chromatography the method employs a simple one-stage extraction step. Nitroglycerin and its dinitrate metabolites are then chromatographed on a 10-μm nitrile bonded phase column using an internal-external standard method. The nitroglycerin and its 1,2,3-propanetriol-1,3- and -1,2-dinitrate metabolites (glyceryl-1,3- and -1,2-dinitrate) have a retention time of 8.5, 10.5, and 11.5 min, respectively at a flow rate of 2.0 mL/min for a mobile phase of 5% v/v acetone in *n*-hexane. The limits of sensitivity were 0.05 ng/mL for nitroglycerin and 0.25 ng/mL for the dinitrate metabolites. Linearity of response was observed over the 0.1–2.0-ng/mL range for nitroglycerin and 0.5–10.0-ng/mL range for the dinitrate metabolites. Blood level data from a pilot study with human volunteers in receipt of an oral form of nitroglycerin is presented.

Keyphrases □ Nitroglycerin—HPLC, parent drug, dinitrate metabolites, human plasma □ Thermal energy analyzer—HPLC, nitroglycerin, dinitrate metabolites, human plasma

Nitroglycerin has been shown to be an effective vasodilator used prophylactically in the treatment of angina (1). The majority of published methods for the analysis of nitroglycerin in plasma employ GC with electron-capture detection (ECD) (2–4). These sensitive methods require complicated extraction procedures and careful handling to ensure reproducibility.

A recent publication (5) describes a very sensitive and rapid assay employing negative ion GC–MS; however ionization techniques are not suitable for analyses of large routine workloads due to continuous contamination of the ion source. A high-performance liquid chromatographic (HPLC) procedure with a thermal energy analyzer as detector has been reported for determining nitroglycerin and its metabolites in beagle dog blood (6). This method has been criticized (7) as being insufficiently sensitive for the determination of the compound in humans following therapeutic doses.

Our laboratories have successfully used HPLC combined with the thermal energy analyzer for the analysis of isosorbide dinitrate in human plasma (8). This report describes the development of a highly sensitive and robust procedure for the assay of nitroglycerin and its dinitrate metabolites in human plasma using similar techniques.

EXPERIMENTAL SECTION

Materials and Apparatus—Pure authentic trinitroglycerin[1] and isosorbide dinitrate[2] were used as received without purification as a standard and internal standard, respectively. 1,2,3-Propanetriol-1,2-dinitrate (glyceryl-1,2-dinitrate)[3] and 1,2,3-propanetriol-1,3-dinitrate (glyceryl-1,3-dinitrate)[3] were supplied as 10% dispersions in cornstarch and purified by extraction with a dichloromethane–ethyl acetate (1:1, v/v) solution before use. All chemicals used were Analar[4] grade. All solvents used were glass distilled[5] or HPLC[5] grade.

The HPLC pump[6] was fitted with a loop injector[7] with a 100-μL loop. The system was interfaced to a thermal energy analyzer[8] with the following conditions: furnace temperature, 575°C; argon flow rate, 15 mL/min; oxygen flow rate, 25 mL/min; range × 8. The slush bath of the instrument consisted of methanol and solid carbon dioxide pellets and was maintained at −77°C. The signal was recorded on a 10.0-mV recorder[9].

A 25-cm × 0.46-mm i.d. column containing a polar bonded-phase packing material[10], was employed at ambient temperature. The material is uniformly spherical, and each particle is ~6 μm in diameter. The mobile phase was prepared by mixing acetone and *n*-hexane in a ratio of 5:95. The solution was deaerated in an ultrasonic bath[11] and was purged with pure helium immediately prior to use. The mobile phase was pumped in an isocratic manner through the column at ambient temperature at a flow rate of 2.0 mL/min, giving a back-pressure ≃500 psi. All glassware was treated with a siliconizing agent[12] and allowed to dry prior to use.

Standard Solutions—Solutions of nitroglycerin, glyceryl-1,2-dinitrate, glyceryl-1,3-dinitrate, and isosorbide dinitrate were prepared daily and stored at 4°C. These solutions were prepared as follows; a working standard solution of nitroglycerin was prepared at a concentration of 1 mg/100 mL in *n*-hexane; the internal standard, isosorbide dinitrate, was prepared at a concentration of 10 mg/100 mL in *n*-hexane. Working standard solutions were prepared for isosorbide dinitrate, glyceryl-1,2-dinitrate, and glyceryl-1,3-dinitrate at concentrations of 100 μg/100 mL in *n*-hexane.

Plasma standards were produced by spiking 5.0-mL aliquots of fresh human plasma with appropriate volumes of the plasma standard solutions to give a range of calibrators with concentrations of nitroglycerin at 0, 0.1, 0.25, 0.5, 1.0, 1.5, and 2.0 ng/mL and glyceryl-1,2-dinitrate and glyceryl-1,3-dinitrate at 0, 0.5, 1.0, 2.5, 5.0, 7.5, and 10 ng/mL. These standards were vortex mixed for 30 s and then allowed to stand at 4°C for 15 min to equilibrate.

The range of calibrators were taken through the complete extraction procedure as described below. Calibration curves were constructed by plotting the peak height ratio of nitroglycerin and its dinitrate metabolites to isosorbide dinitrate, the internal standard, *versus* the appropriate concentrations of the compounds. Plasma samples were similarly treated and the peak height ratios referred to the appropriate calibration curve to read the concentration of nitroglycerin and its dinitrate metabolites present.

Method—All blood samples withdrawn from subjects in receipt of nitroglycerin were collected into treated glass syringes and transferred into treated glass tubes containing 250 U of heparin and 25 μL of 0.002 M silver nitrate solution. The tubes were immediately mixed by inversion and centrifuged at 1000×*g* for 10 min at 4°C.

Aliquots (5.0 mL) of the fresh plasma samples were transferred into 25.0-mL glass extraction tubes fitted with screw caps with polytef liners. Working isosorbide dinitrate internal standard solution (10 μL) was added to all tubes giving a concentration of 2.0 ng/mL of internal standard. Dichloromethane–ethyl acetate (1:1, v/v, 15.0 mL) was added to all tubes, which were then capped tightly and shaken mechanically on a horizontal shaker[13] at 250 cycles/min for 5 min. The tubes were then centrifuged at 750×*g* for

[1] 1% Trinitroglycerin solution ART 7753; E. Merck, Darmstadt, F.R.G.
[2] Kindly donated by Reed and Carnrick Inc., N.J.
[3] Kindly donated by Professor A. Beckett, Chelsea College, London.
[4] B.D.H. Ltd., Poole, Dorset, England.
[5] Rathburn Chemicals Ltd., Walkerburn, Peebleshire, Scotland.
[6] L.D.C. Constametric IIIG HPLC Pump; Milton Roy Corp., Fla.
[7] Rheodyne type 7125; Rheodyne Inc., Calif.
[8] Model 502A/LC; Analytical Instruments Division, Thermo Electron Corp., Waltham, Mass.
[9] Rikadenki Mitsui Ltd., Machinery Sales, Chessington, Surrey, England.
[10] Zorbax CN; Dupont Instruments Ltd., Stevenage, Herts., U.K.
[11] Baird and Tatlock Ltd., Romford, Essex.
[12] Surfasil; Pierce and Warriner (U.K.) Ltd., Chester, Cheshire, U.K.
[13] Braun Lab Shaker LSL; FT Scientific Ltd., Tewkesbury, England.

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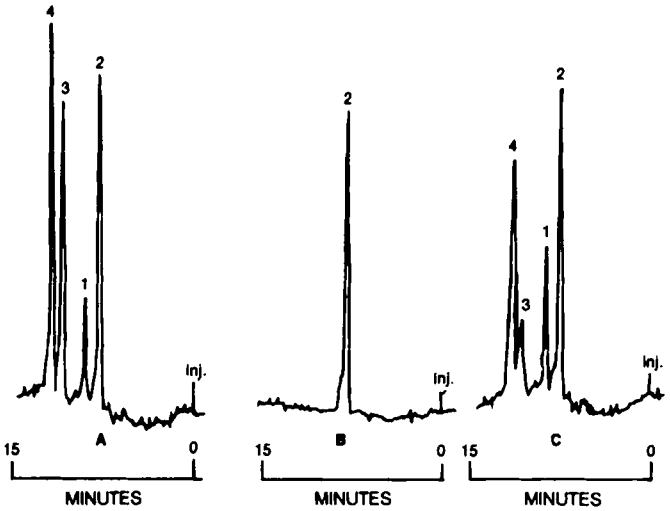

Figure 1—*Determination of nitroglycerin and its metabolites in plasma. Key: (A) spiked test plasma extract containing 0.5 ng/mL of nitroglycerin (1), 2 ng/mL of isosorbide dinitrate internal standard (2), 2.5 ng/mL of glyceryl-1,3-dinitrate (3), and 2.5 ng/mL of glyceryl-1,2-dinitrate (4); (B) spiked plasma extract containing 2 ng/mL of isosorbide dinitrate internal standard (2); (C) plasma extract from a sample withdrawn 15 min following dosing of 2 × 400-μg nitroglycerin sublingual tablets to an adult healthy volunteer indicating the presence of nitroglycerin (1), isosorbide dinitrate (2) (internal standard), glyceryl-1,3-dinitrate (3), and glyceryl-1,2-dinitrate (4).*

5 min at 0°C. The upper aqueous phases were aspirated to waste using a water pump[14] and the lower organic phases were carefully transferred to 20-mL glass vials. The organic phases were then evaporated just to dryness under a gentle stream of nitrogen at room temperature. The vials were capped and stored at −20°C. Immediately prior to analysis on the HPLC, the residues were reconstituted in 150 μL of *n*-hexane.

RESULTS AND DISCUSSION

The chromatographic conditions described were implemented to give the best compromise between chromatographic resolution and peak symmetry and relatively short retention times to permit the analysis of a large throughput of samples with good accuracy and precision. Using these HPLC conditions, isosorbide dinitrate, nitroglycerin, glyceryl-1,3-dinitrate, and glyceryl-1,2-dinitrate give retention times of 7.3, 8.5, 10.5, and 11.5 min, respectively, with a run time between each injection of 15 min.

Figure 1 illustrates a typical plasma chromatogram, chromatograms of spiked plasma samples, drug-free control plasma, and plasma following dosing of a sublingual preparation of nitroglycerin (for comparison). Standard calibration lines were constructed by plotting the peak height ratios of nitroglycerin and its dinitrate metabolites to the isosorbide dinitrate internal standard against the respective concentrations of the compounds. Linearity was found over the standard calibration ranges employed for the parent drug and metabolites. Coefficients of variation of 11.6% at 0.1 ng/mL and 6.6% at 2.0 ng/mL were found for nitroglycerin; comparable data were obtained for the metabolites. The 95% confidence limits obtained for five replicate plasma standard calibrations are shown in Table I and indicate good precision throughout the ranges employed.

Within- and between-batch reproducibility is demonstrated with the following data. Ten replicate plasma control samples were assayed on the same day for each parameter at two levels. Nitroglycerin spiked at 0.4 and 1.0 ng/mL gave mean results of 0.43 (*SD*, ± 0.06) and 1.06 (*SD*, ± 0.18). Between-batch reproducibility on a series of seven control samples for each parameter was 1.7–11.5% (mean, 6.1%) for nitroglycerin, 2.4–14.2% (mean, 6.7%) for glyceryl-1,3-dinitrate, and 3.0–9.0% (mean, 6.1%) for glyceryl-1,2-dinitrate. Spiked pooled plasma samples, when assayed after 7 and 14 d storage at −25°C, appeared not to have deteriorated on storage. Nitroglycerin determined in duplicate samples giving initial mean values of 0.26 and 0.78 ng/mL gave a mean value of 0.22 and 0.66 ng/mL at 7 d and 0.22 and 0.77 ng/mL at 14 d. Glyceryl-1,3-dinitrate and glyceryl-1,2-dinitrate were spiked at concentrations of 1.75 and 3.45 ng/mL and also proved to be stable over the 14-d period. The mean overall recoveries for nitroglycerin and its dinitrate metabolites were calculated over the calibration ranges by comparing peak height ratios for unextracted standards to those taken through the extraction procedure and corrected for the recovery of the internal standard. The recoveries were 76.4% for nitroglycerin, 70.2% for glyceryl-1,3-dinitrate, and 77.0% for glyceryl-1,2-dinitrate.

[14] Filter pump; Baird and Tatlock Ltd.

Table I—Confidence Limits of the Analysis of Spiked Plasma Standards for Nitroglycerin and Its Dinitrate Metabolites

Nitroglycerin		Glyceryl-1,2-Dinitrate		Glyceryl-1,3-Dinitrate	
ng/mL	95% Limits	ng/mL	95% Limits	ng/mL	95% Limits
0.1	0.02	0.5	0.04	0.5	0.07
0.25	0.018	1.0	0.04	1.0	0.09
0.5	0.08	2.5	0.14	2.5	0.31
1.0	0.07	5.0	0.12	5.0	0.35
1.5	0.14	7.5	0.35	7.5	0.45
2.0	0.18	10.0	0.41	10.0	0.27

The minimum detectable levels of nitroglycerin and the 1,3- and 1,2-dinitrate metabolites, determined as the lowest levels discernible over a baseline signal-to-noise ratio of 2:1, were 50, 250, and 250 pg/mL, respectively. These data demonstrate that the developed procedure is sufficiently sensitive and robust for the investigation of the pharmacokinetics of nitroglycerin in humans and further, for the first time, data on two important metabolites of the parent compound can be studied in humans.

Figure 2 illustrates plasma concentration–time profiles representing mean data obtained from 13 subjects in receipt of 800 μg (2 × 400 μg) of nitroglycerin as sublingual tablets. The data on nitroglycerin and its dinitrate metabolites is accompanied by the 95% confidence limits for the individual subject data at each time point. It can be seen that the dinitrate data can prove to be useful in monitoring the elimination of formulations which produce low plasma nitroglycerin concentrations. We have undertaken a preliminary investigation of oral controlled-release formulations and have found that the levels of nitroglycerin are extremely low, in most cases indiscernible from the base line, and yet substantial absorption has occurred as demonstrated by the levels of the two dinitrate metabolites. These findings are contradictory to data obtained by GC–ECD methods (9), where the metabolites are not measured and no data may be available to confirm the specificity of those procedures with regard to the dinitrates.

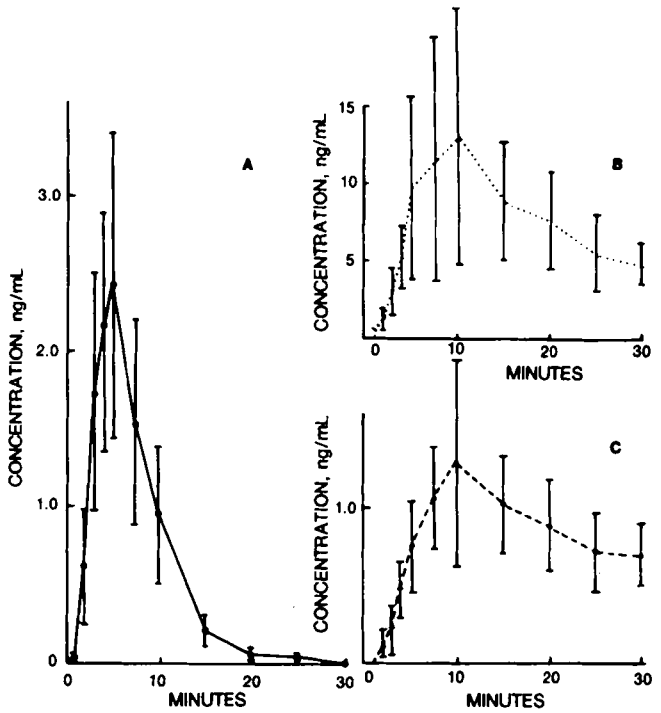

Figure 2—*Mean concentration–time plasma curves and 95% confidence limits for nitroglycerin (A) and its metabolites glyceryl-1,2-dinitrate (B) and glyceryl-1,3-dinitrate (C), for 13 subjects in receipt of 2 × 400-μg sublingual nitroglycerin tablets.*

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Antifungal Properties of 2-*n*-Alkyn-1-ols

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Received September 19, 1983, from the **Boyce Thompson Institute for Plant Research at Cornell University, Ithaca, NY 14853* and the ‡*Departments of Pharmacology and Physiology and* §*Microbiology, School of Dental Medicine, University of Pittsburgh, Pittsburgh, PA 15261.* Accepted for publication March 12, 1984.

Abstract □ Fourteen 2-*n*-alkynols (C_3-C_{14}, C_{16}, and C_{18}) were tested against *Aspergillus oryzae*, *Aspergillus niger*, *Trichoderma viride*, and *Myrothecium verrucaria* in Sabouraud dextrose agar at pH 5.6 and 7.0. Toxicity to *Candida albicans*, *Candida tropicalis*, *Trichophyton mentagrophytes*, and *Mucor mucedo* was determined in the same medium at pH 5.6 and 7.0 in the absence and presence of 10% beef serum. Fungitoxicity was strongly influenced by chain length, slightly by the pH of the medium, and significantly by the presence of beef serum. 2-*n*-Undecyn-1-ol was the most active member of the series, and there was marked synergism between it and ketoconazole.

Keyphrases □ *n*-2-Alkyn-1-ols—*in vitro* antifungal activity, effect of chain length, pH, adsorbents □ Antifungal activity—*n*-alkynols, *in vitro*, effect of chain length, pH, adsorbents □ Structure-activity relationships—*n*-alkynols, antifungal activity, *in vitro* □ Synergism—2-undecyn-1-ol and ketoconazole, *in vitro*, *Candida albicans* and *Candida tropicalis*

In our studies of the effect of structural modification on the fungitoxicity of alkanoic acids (1), 2-alkenoic acids (2), 2-alkynoic acids (3), 2-fluoroalkanoic acids (1), 2-bromoalkanoic acids (2), α,ω-alkanedicarboxylic acids (4), alkoxyacetic acids (5), and *n*-alkanols (6), two major physical factors were observed to influence the activities of the toxicants. These included the partition coefficient and absence or presence of adsorbents such as albumin in the growth medium. Factors that affect partition coefficients of the acids include chain length, pK_a, and pH of the medium (3). The pH of the medium had no significant effect on the activity of the alcohols, and for increased fungitoxicity, a lipophilic substituent at the terminal end of the alkanol is useful (6).

Since the alkynoic acids (3) were considerably more toxic to fungi than the alkanoic acids (1), it was of interest to compare the alkanols (6) with an analogous series of alkynols. It was reported that 1-hydroxy-2-nonyn-4-one, a metabolite from *Ischnoderma benzoinum* (Wahl.) Karst had antifungal activity (7). Also of interest was the observation that alcohol oxidase was irreversibly inhibited by propargyl alcohol and 1,4-butynediol, acetylenic alcohols (8).

The present work is concerned with a systematic evaluation of the homologous series of 2-*n*-alkyn-1-ols of chain lengths C_3-C_{14}, C_{16}, and C_{18} against eight fungi: *Aspergillus oryzae*, *Aspergillus niger*, *Trichoderma viride*, *Myrothecium verrucaria*, *Candida albicans*, *Candida tropicalis*, *Trichophyton mentagrophytes*, and *Mucor mucedo*. Since synergism was demonstrated between ketoconazole, a promising antimycotic agent (9), and amphotericin B and other fungitoxicants (10), it was also desired to determine if there would be any synergism between the most active of the alkynols and ketoconazole.

EXPERIMENTAL SECTION

Some compounds were obtained from commercial sources[1]. Literature methods were used for the preparation of the remaining alkynols: C_{12} (11), C_{14} (12), and C_{16} and C_{18} (13).

The test fungi included *A. oryzae* (ATCC 1101), *A. niger* (ATCC 1004), *T. viride* (ATCC 8678), *M. verrucaria* (ATCC 9095C), *C. albicans* (ATCC 10231), *C. tropicalis* (ATCC 9741), *T. mentagrophytes* (ATCC 9129), and *M. mucedo* (ATCC 7941) (14)[2]. The compounds were tested against *A. oryzae*, *A. niger*, *T. viride*, and *M. verrucaria* in Sabouraud dextrose agar[3] at pH 5.6 and 7.0 according to published methods (1). Graded levels of test compound dissolved in dimethyl sulfoxide (Me_2SO) were incorporated into the growth medium which was subsequently inoculated with the respective fungus. The inoculum was one drop of spore suspension. The preparation of spore suspensions of *A. niger*, *T. viride*, *M. verrucaria*, and *A. oryzae* was accomplished by growing the fungi on potato dextrose agar[3] for several weeks at 28°C in flat, wide-mouth bottles until extensive sporulation had occurred. The spores were harvested by adding 5 mL of sterile 0.85% NaCl solution to the bottles together with ~10 sterile beads, 5 mm in diameter. The spores were freed of the mycelia by shaking, and the suspension was transferred to sterile test tubes and counted in a hemocytometer. The spore concentration was adjusted to 6×10^6 spores/mL by diluting with sterile saline. Inoculations were carried out with Pasteur pipets which deliver 40 drops/mL. Incubation took place at 28°C for 5 d.

For *T. mentagrophytes*, *M. mucedo*, *C. albicans*, and *C. tropicalis* previously described methods were employed (15). Graded levels of test compound

[1] The C_3, C_5-C_{11}, and C_{13} 2-alkyn-1-ols were purchased from Farchan Labs, Willoughby, Ohio, and the C_4 alkynol was obtained from Aldrich Chemical Co., Milwaukee, Wis. Ketoconazole is a product of Janssen Pharmaceutica Inc., New Brunswick, N.J.

[2] These authors present evidence that ATCC 7941 is *Mucor cirinelloides*. We will retain the ATCC nomenclature until it is changed by the American Type Culture Collection.

[3] Difco Labs, Detroit, Mich.

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