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Note

Methods for the quantitation of nitroglycerin and its metabolites in human plasma

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The assay that is described in this study was developed with two objectives in mind: its precision and sensitivity had to be such that a quantitation of nitroglycerin could be accomplished at blood levels found clinically, and that there had to be a separation and quantitation of the major metabolites of the drug, that is, of 1,2-dinitroglycerin and 1,3-dinitroglycerin. No methods presently available meet these objectives.

The blood concentrations that have to be measured are exemplified by the blood levels of nitroglycerin found following sublingual dosing at 0.3 mg, 0.1—1 ng/ml [1], and by the concentrations that are believed to be therapeutic, 1.2—11.1 ng/ml [2]. With respect to our second objective, a concomitant measure of metabolite blood levels can lead to the elucidation of possible tolerance mechanisms, a determination of nitroglycerin's metabolic paths and rate and equilibrium parameters, and a study of the interactions of nitroglycerin with blood proteins and cell components [3]. The interest of these studies in the quantitation of dinitro metabolites stems from the observation that they are the major metabolites of nitroglycerin in man since only small amounts of the other metabolites (mononitroglycerins and glycerol) form [4, 5].

The thin-layer chromatographic (TLC) separation and quantitation of radiolabelled organic nitrates has the requisite resolution but not the needed sensitivity to meet our objectives [5—7]. High-performance liquid chromatographic (HPLC) systems have been reported for the separation of nitroglycerin and its metabolites but the sensitivity possible even with thermal energy analyzer detection is inadequate for biological systems [8].

Gas chromatography (GC) has been the most popular method of assay for nitroglycerin. Although thermal decomposition of organic nitrates is a long

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recognized problem [9, 10] quantitative data are attainable with careful experimental design [11]. Workers primarily interested in the assay of nitroglycerin and not its metabolites have used a variety of GC systems with electron-capture detection for maximum sensitivity. Rosseel and Bogaert [12] using 3.5% QF-1 as stationary phase achieved a sensitivity of 0.5 ng/ml for nitroglycerin using an ethyl acetate extraction of plasma. Their technique was modified by Yap et al. [13] who used a hexane extraction of plasma since it is a better solvent for nitroglycerin. They used a column of 3% SP-2401 and reported a sensitivity of 0.1 ng/ml with about a 20% error. Since hexane is not a good solvent for dinitroglycerins or mononitroglycerins, the modification by Yap et al. [13] gives only qualitative estimates of dinitro content and does not separate the 1,2- and 1,3-dinitroglycerin isomers. Wei and Reid [14] used a hexane extraction and a 30% SE-30 stationary phase to obtain a sensitivity and reproducibility of 0.5 ng/ml (100% error). They reported that the advantages of their system are its simple extraction procedure, its speed, and no extensive solvent cleanup as required by the Yap et al. [13] or Rosseel and Bogaert [12] methods.

In the previously discussed GC methods no serious attempt was made to separate and quantitate the metabolites. However, other studies report efforts in this direction. Armstrong et al. [15] used a double extraction method to detect the dinitro compounds in the presence of nitroglycerin. The column they used, 10% OV-101 on Chromosorb 750, would not separate the parent compound from its dinitro metabolites (retention times 2.4 and 2.8 min). Thus, by first removing the nitroglycerin through hexane extraction and then extracting with diethyl ether to remove the dinitroglycerins, it was possible to detect a mixture of the dinitro compounds but not possible to separate 1,2- from 1,3-dinitroglycerin. Since hexane may also extract metabolites [13], the sensitivity and the accuracy of the procedure must suffer. Rosseel and Bogaert [16] used two columns to separate and identify nitroglycerin and its major metabolites. Using 3.5% QF-1, they obtained a separation of nitroglycerin from a mixture of the dinitroglycerins which had essentially the same retention time. On a 3% XE-60 column, nitroglycerin and 1,2-dinitroglycerin had the same retention time while 1,3-dinitroglycerin was separated. Neurath and Dünger [17] separated nitroglycerin (retention time 9.4 min) from a mixture of dinitroglycerins (retention time 3.1 min) and a mixture of mononitroglycerins (retention time 1.8 min) by derivatizing the organic nitrates and separating them on an OV-17 column. Recently Wu et al. [11] described the characteristics of HPLC systems that successfully separate nitroglycerin from all four metabolites (1,2- and 1,3-dinitroglycerin and 1- and 2-mononitroglycerin) but as with other HPLC systems, the requisite sensitivity for use in physiological systems is lacking. These same studies describe a GC system using a short 3% Carbowax 20M-TPA column to successfully separate nitroglycerin, 1,2- and 1,3-dinitroglycerin in about 10 min.

The method described in the current study combines our own experience [3, 11] with the experience of those workers described above, to optimize the assay of nitroglycerin and its major metabolites. The method essentially uses an extraction of plasma with pentane to maximize nitroglycerin extraction and minimize extraction of interfering materials from plasma. Extraction

of another portion of plasma with ethyl acetate optimizes extraction of dinitro compounds. Both of these samples are chromatographed in a GC system that separates and quantitates all three compounds on a short and highly loaded stationary phase to minimize thermal decomposition.

EXPERIMENTAL

All reagents used were analytical grade unless otherwise indicated. The extraction solvents, pentane and ethyl acetate (for pesticide residue analysis), were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). The gas chromatographic internal standard was *o*-iodobenzyl alcohol (Aldrich, Milwaukee, WI, U.S.A.). Nitroglycerin was purchased from ICI America (Wilmington, DE, U.S.A.) as a 10% adsorbate on lactose. The drug was obtained by alcohol extraction of the adsorbate. It was purified by extracting an aqueous solution of the drug with pentane, separating the supernatant, and removing the pentane over nitrogen. After redissolving the nitroglycerin in water, the procedure was repeated twice. The purity of the nitroglycerin was determined by HPLC and TLC and its solution standardized by a colorimetric assay [18]. Authentic samples of 1,2-dinitroglycerin and 1,3-dinitroglycerin were synthesized from 1,2-dibromopronane-3-ol and 1,3-dibromopropane-2-ol, respectively (both from Eastman Kodak, Rochester, NY, U.S.A.) using the method of Dunstan et al. [19]. The synthesized organic nitrates were isolated using a silica column with an ethyl acetate—benzene mixture as eluent. Identity was determined by nitrate [18], IR [20], TLC [6], and GC [16] analyses. Ninety-nine percent purity was found using a TLC [6] and an HPLC [11] method.

In these studies a Varian 3700 dual-column gas chromatograph (Varian, Palo Alto, CA, U.S.A.) equipped with an 8 mCi ^{63}Ni electron-capture detector was used in conjunction with a Shimadzu Chromatopac C-RIA recording data processor (Shimadzu Scientific Instruments, Columbia, MD, U.S.A.). The column (50 cm × 2 mm silanized glass) consisted of 6% OV-351 (Analabs, North Haven, CT, U.S.A.) on a 90—100 mesh Anakrom ABS (Analabs). The injection port, column, and detector temperatures were 150°C, 136°C, and 200°C, respectively. In order to define the conditions through which retention times may be replicated, a flow-rate of 27 ml/min of 5% methane in argon was used for nitroglycerin and 20 ml/min for the dinitro compounds.

Assays were conducted as follows. Using various volumes of a standard solution of nitroglycerin added to 3 ml of organic solvent (pentane) concentrations of the drug ranging from 50 pg/ml to 110 ng/ml were prepared. Internal standard was added to each solution at a concentration of 0.2, 2.0 or 15 ng/ml depending on the nitroglycerin concentration. These solutions were used as references to determine extraction efficiency as described later. The same volumes of standard solution were transferred to 3 ml of plasma (obtained from the Blood Bank of The Ohio State University Hospitals, Columbus, OH, U.S.A.) giving the same concentration range as the organic reference solutions. Internal standard was added to each plasma sample. The plasma was extracted with 4 ml of pentane by first gently shaking for 4 min followed by centrifugation for 10 min. The organic phase was transferred to a 15-ml conical centrifuge tube. Pentane extraction of the plasma was repeated

and the combined extracts evaporated to near dryness under a nitrogen stream. The residue was reconstituted to 50 μl with pentane and 5 μl were injected into the gas chromatograph. In the analysis of 1,2- and 1,3-dinitroglycerin, the same procedure was used except that ethyl acetate was the solvent used, 3 μl were injected into the column, and no internal standard was used. An external standard method was used for quantitation as described below.

Linearity of the detector was verified over the concentration range used for both nitroglycerin and its metabolites. In the internal standard method for nitroglycerin the ratio of GC area of nitroglycerin to internal standard was plotted as a function of nitroglycerin concentration to generate standard curves. These curves were used to calculate nitroglycerin content at all concentrations used. Extraction efficiencies were obtained by comparing ratio of areas (nitroglycerin to iodobenzyl alcohol) in an internal standard method for nitroglycerin and also using an external standard method, by comparing absolute areas for nitroglycerin or metabolite obtained in the plasma extraction studies with those obtained using the solutions where identical amounts of nitroglycerin (or metabolites) were added directly to organic solvent and subsequently identically treated as the plasma extracts (i.e. evaporation, reconstitution, etc.).

In addition to the internal standard method, analysis for nitroglycerin and metabolites was also conducted using an external standard method. The latter method was the only one used for the dinitroglycerins. In their analyses, fixed amounts of dinitroglycerins were added to known volumes of ethyl acetate extracts of plasma. These were concentrated, reconstituted, injected onto the column, and the standard curves constructed from the areas under the chromatograms obtained. Compound content and precision for the several samples used were determined using these standard curves.

RESULTS AND DISCUSSION

A pentane extraction of normal human plasma followed by concentration and injection into the gas chromatograph gives a chromatogram typified by Fig. 1A. Analysis of plasma containing nitroglycerin, using the method described, results in a retention time of 4.2 min for the drug. Fig. 1B shows the chromatogram obtained in the analysis of plasma containing 0.2 ng/ml nitroglycerin. The internal standard, *o*-iodobenzyl alcohol, is found to have a retention time of 5.3 min and is totally resolved from nitroglycerin. Fig. 1C is the chromatogram obtained for nitroglycerin at 1 ng/ml in plasma containing internal standard at 2 ng/ml. Standard curves for nitroglycerin in three ranges studied (0.1—1, 1—10, 10—110 ng/ml) show excellent linearity. For example in the low and high range the ratio of areas (nitroglycerin/*o*-iodobenzyl alcohol) is related to nitroglycerin concentration with correlation coefficients of 1.0000 and 0.9996, respectively. The extraction efficiency for nitroglycerin is 89% which represents the average for all values in the concentration ranges studied (n = 16). The precision attainable for nitroglycerin is ± 9.1% (n = 4) at 0.2 ng/ml, ± 8.2% (n = 4) at 2 ng/ml, and ± 4.6% (n = 4) at 20 ng/ml. The lower limit for nitroglycerin assay is taken as that level where a precision of about 15% is possible. Hence, the lower limit for

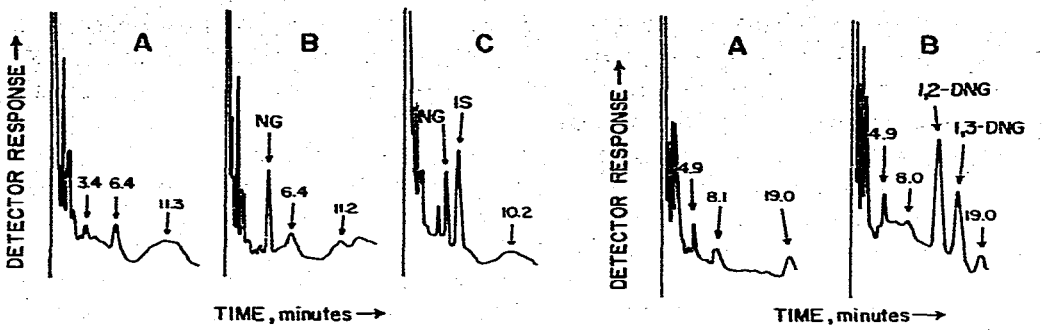

Fig. 1. Gas chromatograms obtained following the pentane extraction of (A) plasma, (B) plasma with 0.2 ng/ml nitroglycerin, and (C) plasma with 1 ng/ml of nitroglycerin and 2 ng/ml of internal standard. Peaks: NG = nitroglycerin (retention time 4.2 min); IS = internal standard, *o*-iodobenzyl alcohol (retention time 5.3 min).

Fig. 2. Gas chromatograms obtained following (A) ethyl acetate extraction of plasma, and (B) plasma containing 50 ng/ml of 1,2-dinitroglycerin and 40 ng/ml of 1,3-dinitroglycerin. Peaks: 1,2-DNG = 1,2 isomer (retention time 12.8 min); 1,3-DNG = 1,3 isomer (retention time 15.6 min).

nitroglycerin using this method is 50 ± 8.2 pg/ml (n = 3). The level of precision at this low concentration is the best among assays for nitroglycerin currently being used.

Ethyl acetate extraction of plasma can lead to chromatograms that are erratic, ranging from chromatograms that are relatively uncomplicated to ones that are replete with interfering peaks. Fig. 2A shows the chromatogram obtained for a plasma blank and Fig. 2B shows the chromatogram for the same plasma to which 1,2-dinitroglycerin (50 ng/ml) and 1,3-dinitroglycerin (40 ng/ml) had been added. The retention times for the 1,2- and 1,3-isomers were 12.8 min and 15.6 min, respectively. The average extraction efficiency for the dinitroglycerins was 80.5% (n = 20). It was found that there was no difference in the extraction efficiency of the two isomers and the number thus listed is an average for studies involving both isomers. Using the external standard method, standard curves of area (digital printout of the C-RIA processor) versus concentration showed excellent linearity. Again, both isomers behaved identically. Using these curves, a precision of ± 7.4% (n = 8) was found for the dinitroglycerins at a concentration of 50 ng/ml. The lower limit for their assay was taken as 7.5 ± 1.1 ng/ml (n = 6).

The chromatographic system used will resolve and quantitate nitroglycerin, 1,2-dinitroglycerin, and 1,3-dinitroglycerin. It would be most desirable to be able to extract an aqueous sample with a single solvent and effectively remove all three organic nitrates. Unfortunately, the physical properties of nitroglycerin and its major metabolites are quite different so that the identification of a single solvent suitable for both is difficult. Thus, in the interest of accuracy and sensitivity, it is recommended that the dual sample extraction procedure be used. Pentane extracts nitroglycerin and minimizes the removal of chromatographic interfering materials present in plasma. Ethyl acetate, on the other hand, efficiently extracts the more polar dinitroglycerins and thus optimizes their quantitation.

Thermal decomposition probably occurs on the column as observed in our earlier work [11]. The extent of decomposition could be as much as 12% but it should be minimized through the use of the short, relatively loaded column where adsorption would be less.

Moreover, if it is assumed that the same extent of thermal decomposition occurs with the standards as occurs with the samples at the same concentration, this would correct the samples for any thermal decomposition and quantitation of the organic nitrates is unaffected.

Yap et al. [13] suggest that initial treatment of the plasma with silver nitrate is necessary to slow down the degradation of nitroglycerin in plasma. Using the half-life for nitroglycerin in human plasma at 24°C (12 h) [21] only 1.5% drug would be lost during sample preparation using the method proposed here (15 min). The relative effect of silver nitrate depends on the amount used as does the amount of precipitate formed upon its addition to plasma. The latter most likely affects the accuracy and precision of the assay. It is our feeling that the addition of silver nitrate does not provide any real advantage. These observations of potential instability of nitroglycerin suggest that there is good reason to extract blood directly with organic solvent as soon as the blood sample is collected. Extraction efficiencies might be different in comparison with plasma, but the procedure should reduce any drug loss subsequent to blood collection and before actual drug analysis.

Both internal and external standard methods were used in this study. It seems, however, that the external standard is preferred especially when the levels of drug or metabolite are very low in blood or plasma. The reason for this is the erratic and unpredictable presence of interfering materials extracted from biological samples that subsequently appear in the chromatogram. These interfering peaks vary in position from person to person and within a particular indivual's plasma at different times. Their presence makes the choice of an internal standard with an appropriate retention time difficult.

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