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Report

Capillary Gas Chromatographic (GC) Analysis of Nitroglycerin and Its Denitration Products in Plasma

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A convenient, specific, and sensitive capillary gas chromatographic (GC) assay for analyzing nanogram concentrations of nitroglycerin and its dinitro- and mononitrometabolites in plasma has been developed. Using a bonded-phase (DB-1) 30-m, 1-μm-thick film capillary column and a 1-m, 5-μm-thick film precolumn, separation of nitroglycerin and all four partially nitrated metabolites was achieved in less than 15 min. On-column injection, electron capture detection, and isothermal operation at 100°C yielded a linear extraction curve over a 300-ng/ml range without any need to concentrate sample extracts. Using methyl *t*-butyl ether as extraction solvent and *o*-chloronitrobenzene as internal standard, recoveries from plasma spiked at levels greater than 10 ng/ml approximated 35% for the 1-monometabolite, 40% for the 2-monometabolite, and greater than 90% for all others. The method was employed in a pharmacokinetic study of nitroglycerin administered intravenously to beagle dogs. Plasma samples were collected at various time points and analyzed.

KEY WORDS: nitroglycerin; metabolites; capillary gas chromatography with electron capture detection (GC-EC); nanogram; beagles.

INTRODUCTION

Nitroglycerin (GTN) has been used for over 100 years to treat angina (1) and is currently available in sublingual capsules, buccal controlled-release (CR) tablets, ointments, i.v. injectable solutions, transdermal patches, and oral controlled-release formulations. Orally administered GTN undergoes extensive presystemic metabolism. It is rapidly metabolized in the liver to 1,2-dinitroglycerin (1,2-GDN) and 1,3-dinitroglycerin (1,3-GDN), which are further metabolized to 1-monotriglycerin (1-GMN) and 2-mononitroglycerin (2-GMN). Nitroglycerin metabolism by the liver and kidney has been investigated by several groups (2–5); however, a considerable body of evidence also indicates a high rate of metabolism involving red blood cells (6–8).

Gas chromatography employing electron capture detection (GC-EC) is one of the most widely used and sensitive monitors of nitro compounds (9–13). The capillary technique of Noonan *et al.* (9), which employs an extract concentration step, is sufficiently sensitive to quantitate GTN down to the picogram/per milliliter levels seen in transdermal delivery. Packed column techniques are available and adequate for GTN monitoring (11,14), but to date, it has been difficult to detect and quantitate all metabolites by gas chromatography.

High-performance liquid chromatography (HPLC) with UV detection, both normal and reversed phase, has been used to assay oral capsules and tablets (15–18) but has not been sufficiently sensitive for biological assay. However, the use of HPLC with thermal energy analyzer (TEA) detection has been successfully used to monitor GTN and its nitrated metabolites in animals (19) and human plasma samples (20). Glycerol, the penultimate metabolite, is not conveniently assayed by any existing technique used to monitor GTN and is of little interest due to its lack of pharmacological activity. There is evidence of some pharmacological activity exhibited by the dinitrometabolites (3) and the pharmacokinetics of these two compounds has been examined (21–23). There has, however, been no reliable and convenient way to monitor the disposition of all the partially nitrated glycerols in a biological system.

This study reports the development of a sensitive and reproducible capillary GC-EC essay for the identification and quantitation of GTN and all four nitrate metabolites in plasma. Further, the results of preliminary work involving the administration of GTN to beagles are reported.

MATERIALS AND METHODS

Reagents

Nitroglycerin/lactose absorbate (10%) was obtained from ICI Americas, Wilmington, Del. 1,3-GDN, 1,2-GDN,

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1-GMN, and 2-GMN were prepared in our laboratories using a modified method of Dunstan *et al.* (24,25) and purified via preparative HPLC (10). Reference standard solutions of the four metabolites in absolute ethanol, available from Radian Corp., Austin, Tex., were also used. *o*-Chloronitrobenzene and *m*-dinitrobenzene were obtained from Aldrich Chemical Co., Milwaukee, Wis. Potassium carbonate and sodium chloride were obtained from Fisher Scientific Co., Fairlawn, N.J. Solvents methyl *t*-butyl ether, hexane, ether, and ethyl acetate, were either spectro or HPLC grade and were obtained from Burdick and Jackson, Muskegon, Mich. They were used without further purification.

The hydrogen carrier gas (zero grade) and 5% methane/argon makeup gas (certified) were obtained from Roberts Oxygen Co., Rockville, Md. Deionized water was generated with a Milli-RO/ Milli-Q system obtained from Millipore, Bedford, Mass.

Equipment

GC analyses were performed with a Hewlett-Packard Model 5830A chromatograph equipped with a Ni^{63} electron capture detector and electronic integrator. The column was a DB-1 30-m × 0.32-mm-i.d., 1-μm film capillary column from J & W Scientific, Cordova, Calif., fitted with a methyl silicone 1-m × 0.32-mm-i.d., 5-μm film protective precolumn from Quadrex, New Haven, Conn. The on-column injector was a J & W Scientific Model II. Refrigerated centrifugations were performed on a IEC Model 5000 refrigerated low-speed centrifuge.

Chromatographic Conditions

The column was maintained at 100°C (isothermal), and the detector 225°C. The hydrogen carrier gas was maintained at 30-psi column head pressure, and the 5% methane/argon detector makeup-gas maintained at 43 ml/min. Both gases were passed through molecular sieve (5Å) drying cartridges and oxytraps (Alltech Assoc., Deerfield, Ill.). The on-column injector was a telescoping type which allowed for the injection of a 2-μl sample at ambient temperature and subsequent manual insertion of the assembly into the column oven. The injection was made using a gas-tight syringe fitted with a fused silica needle. At the conclusion of each analysis the column oven was ramped to 150°C to purge contaminants, then returned to 100°C for the next injection.

Administration of Drug, Collection and Handling of Samples

Two conscious beagle dogs weighing approximately 10 kg were administered GTN intravenously. Several groups have documented the loss of GTN due to absorption when soft plastic containers were used to deliver solution formulations (26–28). Therefore, the intravenous solution, consisting of Nitrobid i.v. (2.5 mg) diluted to 5 ml with normal saline, was administered via a glass syringe and nonabsorbing PTFE infusion set directly into a forepaw vein (1 to 1.5-min push). Blood samples (8 ml) were collected into heparinized vacutainers (green top) via venipuncture of a neck vein at 5, 10, 15, and 30 min and 1, 1.5, 2, 3, 4, and 24 hr. Blood samples were mixed with heparin, then immediately chilled to 0°C. They were centrifuged at 2000 rpm and 5°C for 15 min and the plasma was separated, frozen, and stored at −35°C until analyzed. A 1-ml portion of plasma was transferred to a round-bottom test tube containing 0.5 g sodium chloride. A 1-ml portion of solvent containing internal standard (*o*-chloronitrobenzene, 12.5 ng/ml) was added, and the tube capped and rocked gently (Thermolyne Speci-Mix) for 15 min. Following centrifugation at 2000 rpm for 5 min the organic layer was transferred to a second round-bottom test tube and dried over 1 g anhydrous potassium carbonate for 3 min. A 2-μl portion was then removed and injected into the gas chromatograph. A new standard curve was run for each set of samples by spiking 1.0-ml aliquots of predosing plasma with known amounts of GTN, GDNs, and GMNs and taking the spiked plasma through the analytical procedure along with the samples. This was necessary since the slope of the standard curve changed with repeated injections due to changes in the characteristics of the bonded phase.

RESULTS AND DISCUSSION

Chromatography

Figure 1 illustrates the separation and resolution of GTN from the GDNs, the GMNs, and the *o*-chloronitrobenzene internal standard. *m*-Dinitrobenzene was also tried as an internal standard; however, its retention time was approximately 15 min—well after that of GTN. Extracted plasma constituents did not interfere since they either eluted at the solvent front, eluted after the peaks of interest, or were not sensitive to EC detection. The 30-m, 1-μm-thick film capillary was protected by a 1-m, 5-μm "very thick film" precolumn. Investigations indicated that repeated injections of solvents containing dissolved water would cause the column to lose progressively its ability to separate 1,2-GDN from 1,3-GDN and 1-GMN from 2-GMN. This loss of resolution was accompanied by increased peak tailing. The peaks for the GMNs were the most adversely affected. However, peaks for the less polar GTN and the internal standard *o*-chloronitrobenzene were little changed. Nonpolar solvents such as hexane, which do not dissolve significant amounts of water, cannot be used since they do not extract the relatively polar GDNs and GMNs. Examination of several drying agents indicated that anhydrous potassium carbonate was rapid and effective. The basic nature of carbonate salts, however, can cause degradation of the nitrate esters upon prolonged contact. For periods up to 1 hr there was little observed degradation and the solution was sufficiently dried to prevent rapid loss of column efficiency. The 5-μm, very thick film precolumn protected the analytical column from the damaging effects of water but required replacement after 35–50 injections.

The J & W Scientific Model II on-column capillary injector employs a stopcock rather than a septum at the column inlet and allows for the withdrawal of approximately 7 in. of column from the oven during injection. The injection is thus made at ambient temprature. By employing this injection system, we were able to inject unusually large sample volumes onto the capillary column (e.g. 2 μl) without the erratic results observed with the original Hewlett Packard "duckbill" column inlet system.

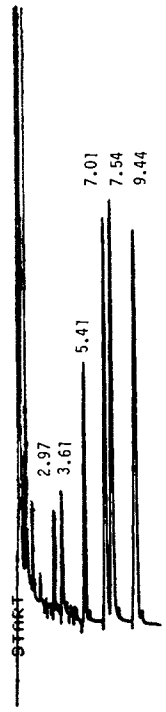

Fig. 1. Chromatogram obtained from the GC analysis of beagle plasma spiked with nitroglycerin and its denitration products. Concentration of each analyte, approximately 50 ng/ml. Peak identification according to retention time: 2.97 min, 1-mononitroglycerin; 3.61 min, 2-mononitroglycerin; 5.41 min, *o*-chloronitrobenzene (internal standard); 7.01 min, 1.3-dinitroglycerin; 7.54 min, 1,2-dinitroglycerin; 9.44 min, nitroglycerin.

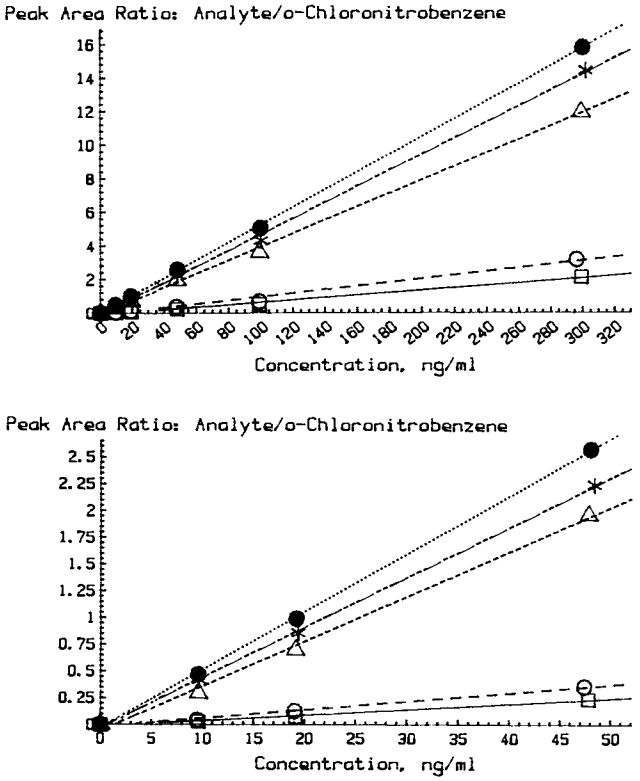

Fig. 2. Calibration curves for nitroglycerin (●), 1,3-dinitroglycerin (△), 1,2-dinitroglycerin (*), 1-mononitroglycerin (□), and 2-mononitroglycerin (○). Standards were extracted from beagle plasma. The lower plot is an expanded scale near the origin.

Samples

Reports of rapid GTN hydrolysis in whole blood (6–8) prompted us to chill samples in an ice bath immediately after collection and further rapidly centrifuge and separate the plasma at 5°C or below. However, we observed that plasma samples stored at −35°C could undergo at last two separate thaw and extraction cycles with no significant difference in measured levels of GTN. Further, no significant loss of parent compound or metabolites was observed in plasma stored at room temperature for short periods of time (e.g., 30 min). Literature reports indicate plasma hydrolysis rates for the conversion of GTN to GDN to be ($t_{1/2}$ = 3 hr) 8- to 10-fold slower than those in whole blood ($t_{1/2}$ = 15–32 min) (6,8). The GDN-to-GMN conversion is very slow in plasma.

Several extraction solvents were examined. Optimum recoveries were obtained using methyl *t*-butyl ether: above 10 ng/ml, GTN and the GDNs were extracted with 92–109% extraction efficiencies, and the more polar 1-GMN and

Table I. Plasma Recovery Data

Spiked concentration of each analyte (ng/ml)	Extraction efficiency, %[a] (CV)[b]				
	1-GMN	2-GMN	1,3-GDN	1,2-GDN	GTN
0	—[c]	—[c]	—[c]	—[c]	—[c]
10	44 (NA)[d]	61 (9.1)	60 (7.8)	105 (4.7)	83 (12.3)
20	34 (8.2)	49 (1.8)	99 (1.7)	101 (0.3)	93 (1.3)
50	33 (8.6)	40 (8.4)	109 (4.8)	103 (4.1)	102 (3.0)
100	30 (10.9)	32 (13.0)	93 (6.1)	92 (9.4)	95 (7.7)
300	30 (7.8)	45 (6.2)	102 (2.2)	101 (1.0)	100 (1.1)

[a] Extraction solvent volume/plasma volume = 1/1.
[b] Coefficient of variation; N = 3.
[c] Below the limit of detection: GTN and GDNs, 0.001 ng on column; GMNs, 0.002 ng on column.
[d] N = 2; coefficient of variation not available.

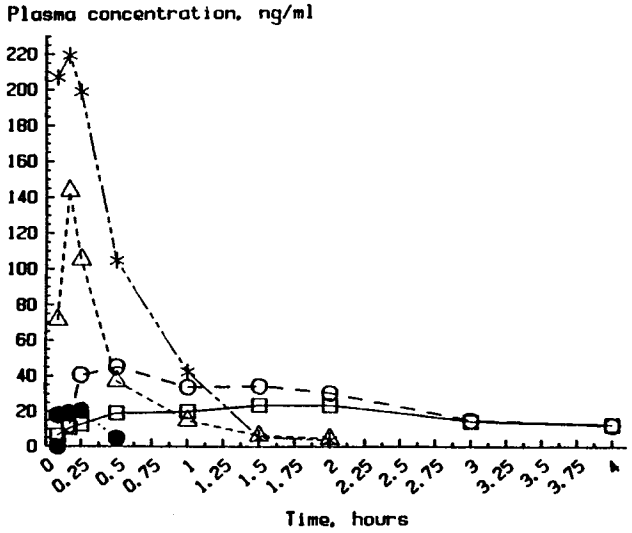

Fig. 3. Plasma concentration of nitroglycerin (●), 1,3-dinitroglycerin (△), 1,2-dinitroglycerin (*), 1-mononitroglycerin (□), and 2-mononitroglycerin (○) following i.v. administration (1-min push) of 2.5 mg to a male beagle.

2-GMN with 30–34% and 32–49% extraction efficiencies, respectively. The results are presented in Table I. Other solvents such as ethyl ether and ethyl acetate extracted GTN and the GDNs with approximately 80 and 85% extraction efficiencies, respectively. The extraction efficiency for the GMNs was not determined with these solvents.

The assay has a practical minimum quantitation level of 10 ng/ml for GTN and the metabolites. Responses are linear from 10 to 300 ng/ml and typical coefficients of variation (CV) are shown in Table I. Typical standard calibration curves are shown in Fig. 2.

Figure 3 illustrates the plasma levels of GTN and its four nitrated metabolites for 4 hr following i.v. administration of GTN. GTN dropped below detectable limits in 30 min. The GDNs peaked at 10 min but persisted for 1.5 hr. The GMNs were evident for up to 4 hr, but at lower concentrations than those observed for the dinitrometabolites.

In summary, we have developed a specific and sensitive GC-EC assay for measuring GTN and all four partially nitrated metabolites. The utility of the assay has been demonstrated in preliminary studies involving the intravenous administration of GTN to dogs.

This methodology has been used in a bioequivalency study of several different oral CR GTN products, the results of which will be presented later.

REFERENCES

1. J. R. Parratt. *J. Pharm. Pharmacol.* 31:801–809 (1979).
2. P. Needleman, S. Lang, and E. M. Johnson, Jr. *J. Pharmacol. Exp. Ther.* 181:489–497 (1972).
3. P. Needleman. *Annu. Rev. Pharmacol. Toxicol.* 16:81–93 (1976).
4. A. T. Blei, J. Gottstein, and H.-L. Fung. *Biochem. Pharmacol.* 33:2681–2686 (1984).
5. M. H. Litchfield. *J. Pharm. Sci.* 60:1599–1607 (1971).
6. P. K. Noonan and L. Z. Benet. *Int. J. Pharm.* 12:331–340 (1982).
7. L. S. Wu, D. Smith, A. M. Burkman, and T. D. Sokoloski. 35th Natl. Meeting APhA/APS, Miami Beach, Fla., Nov. 13–17, 1983.
8. J. A. Armstrong, S. E. Slaughter, G. S. Marks, and P. W. Armstrong. *Can. J. Physiol. Pharmacol.* 58:459–462 (1980).
9. P. K. Noonan, I. Kanfer, S. Riegelman, and L. Z. Benet. *J. Pharm. Sci.* 73:923–927 (1984).
10. A. Carlin, V. K. Prasad, J. E. Simmons, and J. P. Skelly. 39th Natl. Meeting APhA/APS, Minneapolis, Minn., Oct. 20–24, 1985. Abstr. 37, PAC Sect.
11. P. S. K. Yap, E. F. McNiff, and H.-L. Fung. *J. Pharm. Sci.* 67:582–583 (1978).
12. M. T. Rosseel and M. G. Bogaert. *J. Chromatogr.* 64:364–367 (1972).
13. M. T. Rosseel and M. G. Bogaert. *J. Pharm. Sci.* 68:659–660 (1979).
14. C. C. Wu, T. D. Sokoloski, A. M. Burkman, and L. S. Wu. *J. Chromatogr.* 228:333–339 (1981).
15. C. C. Wu, T. D. Sokoloski, A. M. Burkman, M. F. Blanford, and L. S. Wu. *J. Chromatogr.* 216:239–249 (1981).
16. D. M. Baaske, J. E. Carter, and A. H. Amann. *J. Pharm. Sci.* 68:481–483 (1979).
17. W. C. Yu and E. U. Goff. *Biopharm. Drug Disp.* 4:311–319 (1983).
18. C. S. Olsen and H. S. Scroggins. *J. Pharm. Sci.* 72:963–965 (1985).
19. R. J. Spanggord and R. G. Keck. *J. Pharm. Sci.* 69:444–446 (1980).
20. A. J. Woodward, P. A. Lewis, M. Aylward, R. Rudman, and J. Maddock. *J. Pharm. Sci.* 73:1838–1840 (1984).
21. F. N. Lee, N. Watari, and L. Z. Benet. 133rd Ann. Meeting APhA, San Francisco, March 16–20, 1986, Abstr. P-91, PDD Sect.
22. P. S. K. Yap and H.-L. Fung. *J. Pharm. Sci.* 67:584–586 (1978).
23. N. Watari and L. Z. Benet. 39th Natl. Meeting APhA/APS, Minneapolis, Minn., Oct. 20–24, Abstr. 38, PDD Sect.
24. I. Dunstan, J. V. Griffiths, and S. A. Harvey. *J. Chem. Soc.* 1319–1323 (1965).
25. I. Dunstan, J. V. Griffiths, and S. A. Harvey. *J. Chem. Soc.* 1325–1327 (1965).
26. A. H. Scheife, J. A. Grisafe, and L. Shargel. *J. Pharm. Sci.* 71:55–59 (1982).
27. E. F. McNiff, C. M. Lai, Z. M. Look, A. Yacobi, and H.-L. Fung. *J. Pharm. Sci.* 74:774–776 (1985).
28. E. F. McNiff, A. Yacobi, F. M. Young-Chang, L. H. Golden, A. Goldfarb, and H.-L. Fung. *J. Pharm. Sci.* 70:1054–1058 (1981).