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SIMULTANEOUS DETERMINATION OF NITROGLYCERIN AND ITS DINITRATE METABOLITES BY CAPILLARY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

FRANK W. LEE, NOBUTOSHI WATARI, JEAN RIGOD and LESLIE Z. BENET*

Department of Pharmacy, School of Pharmacy, University of California, San Francisco, CA 94143-0446 (U.S.A.)

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SUMMARY

A sensitive gas chromatographic–electron-capture detection method for the simultaneous determination of the antianginal drug nitroglycerin (GTN) and its dinitrate metabolites (1,2-GDN and 1,3-GDN) was developed. Human plasma samples (1 ml) spiked with 2,6-dinitrotoluene as the internal standard were extracted once with 10 ml of a methylene chloride–pentane mixture (3:7, v/v). Using this solvent system, less contaminants are extracted into the organic phase from plasma, resulting in cleaner chromatograms and prolonged column life. A break point was observed on the standard curves of GTN and GDNs. The two linear regions for the detectable concentrations of GTN are 0.025–0.3 and 0.3–3 ng/ml and for 1,2-GDN and 1,3-GDN they are 0.1–1 and 1–10 ng/ml. The limits of detection by this method for GTN, 1,2-GDN and 1,3-GDN in plasma are 0.025, 0.1 and 0.1 ng/ml, respectively.

INTRODUCTION

The development of a specific and sensitive assay method for nitroglycerin (GTN) and its dinitrate metabolites (1,2-GDN and 1,3-GDN) has been a difficult task. The low GTN plasma concentrations observed after therapeutic dose administration [1], adsorption of the drug to plastics [2] and the rapid metabolism of the drug in blood [3,4] make the measurement of GTN difficult. Several assay methods for GTN including gas chromatography (GC) [5–9], high-performance liquid chromatography (HPLC) [10] and gas chromatography–mass spectrometry (GC–MS) [11–14] have been developed, but only a few of these papers describe the assay of the dinitrate metabolites [9,13,14]. The majority of the methods are limited by their lack of low-level detectability, by the large plasma sample volumes needed and by the complexity of the analytical procedure required. In recent years, the more sensitive capillary GC–ECD technique has been

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widely used. In our laboratory, Noonan et al. [15] were able to measure 25 pg of GTN in 1 ml of plasma by using a GC–ECD system equipped with an on-column injector and a fused-silica capillary column. Sioufi and Pommier [8] reported a similar method with a quantitation limit of 50 pg for GTN in 1 ml of plasma. During the course of this work, Noonan et al. [16] and Sioufi et al. [9] reported methods for measurement of the dinitrate metabolites, using a separate analytical procedure from that for GTN.

This paper presents the first selective and sensitive capillary GC–ECD method which is capable of simultaneously measuring picogram levels of GTN, 1,2-GDN and 1,3-GDN in 1 ml plasma, thereby not requiring a second analytical procedure for the metabolites as has been used previously [9,14,16]. Since this assay does not involve GC–MS, it can be readily adopted by any laboratory having capillary GC instrumentation.

EXPERIMENTAL

Chemicals and reagents

GTN (Nitro-Bid) was purchased from Marion Labs. (Kansas City, MO, U.S.A.). Dinitrate metabolites of nitroglycerin (1,2-GDN and 1,3-GDN) were provided by Marion Labs. as pure chemicals (>99%) and were used without further purification. 2,6-Dinitrotoluene was obtained from K&K Labs., ICN (Hollywood, CA, U.S.A.). Methylene chloride (J.T. Baker, HPLC grade) was treated with charcoal and redistilled before use. Butyl acetate and pentane (HPLC grade) were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Instruments

A Varian model 3700 gas chromatograph (Walnut Creek, CA, U.S.A.) equipped with a Varian ^{63}Ni electron-capture detector, a J&W on-column injector (J&W Scientific, Rancho Cordova, CA, U.S.A.) and a Hewlett-Packard Model 3390A integrator (Palo Alto, CA, U.S.A.) were used. An HP-1 fused-silica capillary column (25 m×0.32 mm I.D., 1 μm film thickness) was purchased from Hewlett-Packard. Hydrogen (zero grade, Liquid Carbonic, Chicago, IL, U.S.A.) at a flow-rate of 15 ml/min was used as the carrier gas. Nitrogen (99.997%, Liquid Carbonic) at a flow-rate of 30 ml/min was used as the make-up gas. The column temperature program was set at 96°C initially, held for 9 min, and then increased to 126°C at 4°C/min. After each assay the column temperature was raised rapidly to 280°C and maintained for 5 min to wash out plasma residues.

Sample preparation

All glassware was silanized with a 10% (v/v) dimethyl dichlorosilane solution in toluene to prevent adsorption. After soaking for 30 min in the silanization reagent, glassware was immediately rinsed with toluene and methanol before being dried. Plasma (1 ml) was transferred into a test tube (16×150 mm) containing 100 μl (2 ng) of internal standard (2,6-dinitrotoluene) aqueous solution and known amounts of GTN, 1,2-GDN, and 1,3-GDN, and was immediately extracted

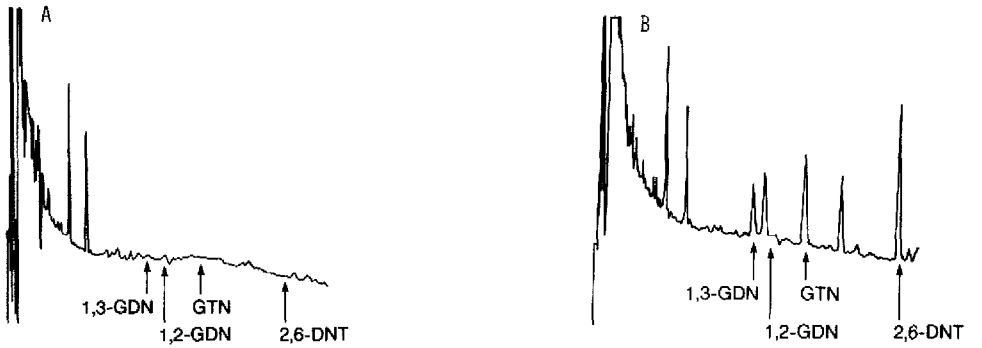

Fig. 1. (A) Gas chromatogram of blank plasma. (B) Gas chromatogram of plasma sample spiked with 1 ng/ml GTN, 1,2-GDN and 1,3-GDN each. 2,6-Dinitrotoluene (2,6-DNT) was used as internal standard. The peak between GTN and 2,6-DNT represents a second internal standard, 2-isosorbide mononitrate, which was also investigated. All analyses described here utilized 2,6-DNT for the internal standard which yielded better reproducibility.

once with 10 ml of methylene chloride–pentane solvent (3:7, v/v). The samples were mixed well on a mechanical rotator (24 rpm) for 20 min and centrifuged at 1500 *g* for 10 min. The organic phases were evaporated under nitrogen at room temperature. The sample vials were removed from nitrogen before they were completely dry. The residue was then reconstituted into 50 μl *n*-butyl acetate. An aliquot of 0.1–0.5 μl was injected onto the gas chromatograph using an on-column injector syringe.

Standard curves

Standard solutions containing GTN, 1,2-GDN and 1,3-GDN were prepared as aqueous solutions. Suitable aliquots were added to test tubes containing 1 ml blank plasma to prepare standard samples. Concentrations of GTN ranged from 0.025 to 10 ng/ml, while concentrations of 1,2-GDN and 1,3-GDN ranged from 0.1 to 10 ng/ml. Fig. 1 shows a typical gas chromatogram of well separated GTN, 1,2-GDN and 1,3-GDN. Calibration curves were obtained by plotting peak-height ratios (nitrates/internal standard, weight $= 1/y^2$) versus plasma concentrations for each of the three compounds.

RESULTS AND DISCUSSION

Instrumentation

It has been reported that nitroglycerin may be adsorbed on active surfaces of capillary injectors, glass columns and detectors [15]. This is also true for glyceryl dinitrate metabolites, making quantification of picogram amounts on conventional GC equipment impossible. Therefore, the on-column injection technique was used to deliver the sample directly into the inert fused-silica capillary column. Optimization of the position of the column exit inside the electron-capture detector is an important factor so as to balance the loss of and detection sensitivity for all three nitrates. The position of the column exit was optimized according

TABLE I

RECOVERY OF METHYLENE CHLORIDE–PENTANE-EXTRACTABLE 1,2-GDN FROM [^{14}C]1,2-GDN-SPIKED HUMAN PLASMA

A 1-ml volume of plasma was used.

Methylene chloride–pentane ratio (v/v)	Extractable radioactivity ($n=2$) (%)	
	Low concentration (0.025 ng/ml)	High concentration (20 ng/ml)
*One extraction**		
50:50	63.2	63.7
40:60	54.3	50.9
30:70	49.5	45.1
Two extractions		
50:50	90.0	84.4
40:60	77.0	75.5
30:70	63.7	66.1
Three extractions		
50:50	85.9	87.2
40:60	93.7	94.5
30:70	89.0	89.6

*A 10-ml volume of solvent was used in each extraction.

to the method reported by Noonan et al. [15]. Four other fused-silica capillary columns coated with dimethylsiloxane were tested for suitability in this assay. The Durabond-1 (DB-1) column from J&W Scientific, the RSL-150 column from Alltech Assoc. (Los Altos, CA, U.S.A.) and the SPB-1 column from Supelco (Supelco Parks, Bellefonte, PA, U.S.A.) exhibited poor resolution and detectability for the three nitrates. The Quadrex-007 column (Quadrex, New Haven, CT, U.S.A.) and the HP-1 column (see Fig. 1) demonstrated good separation and detectability. The HP-1 column was used in all assays reported here and in our experimental studies due to its readily available local supply. Other capillary columns with stationary phases more polar than dimethylsiloxane were also tested and failed to separate 1,2-GDN and 1,3-GDN.

Solvent extraction and recovery

Ethyl acetate, diethyl ether and chloroform were tested as extraction solvents. With these solvents, all three nitrates could be extracted from plasma; however, polar contaminants which interfere with nitrate peaks and shorten column life were also extracted. A mixture of methylene chloride–pentane (3:7, v/v) was found to be a significantly better extraction solvent. The recovery of [^{14}C]1,2-GDN using this extraction procedure was determined, as listed in Table I. The percentage extractable 1,2-GDN was about 50% at low (0.025 ng/ml) and high (20 ng/ml) concentrations when the [^{14}C]1,2-GDN-spiked 1-ml plasma samples were extracted once with 10 ml of methylene chloride–pentane. Although the extractability with the ratio 3:7 mixture was lower than that for ratio 5:5 mix-

TABLE II

WITHIN-DAY PRECISION (REPEATABILITY) AND CALCULATED CONCENTRATIONS

Spiked concentration (ng/ml)	Calculated concentration (mean ± S.D., $n=6$) (ng/ml)	Coefficient of variation (%)
GTN		
0.03	0.031 ± 0.001	3.23
0.1	0.101 ± 0.004	4.24
1	0.994 ± 0.039	3.89
10	9.98 ± 0.07	0.72
1,2-*GDN*		
0.1	0.099 ± 0.005	4.70
1	1.040 ± 0.047	4.58
10	9.82 ± 0.23	2.37
1,3-*GDN*		
0.1	0.101 ± 0.004	4.00
1	1.02 ± 0.05	5.14
10	10.1 ± 0.29	2.87

ture, it was still sufficient to allow measurements of low levels for all three glyceryl nitrates. The ratio 3:7 mixture was chosen since useful column life was maintained significantly longer compared to the 5:5 ratio mixture.

Within-day precision (repeatability)

The within-day precision of this method was checked by analysis of six replicate plasma samples to which known amounts of GTN, 1,2-GDN and 1,3-GDN

TABLE III

BETWEEN-DAY PRECISION (REPRODUCIBILITY) AND CALCULATED CONCENTRATIONS

Spiked concentration (ng/ml)	Calculated concentration (mean ± S.D., $n=6$) (ng/ml)	Coefficient of variation (%)
GTN		
0.03	0.030 ± 0.002	4.97
0.1	0.101 ± 0.002	1.98
1	1.01 ± 0.02	1.86
10	9.93 ± 0.31	3.07
1,2-*GDN*		
0.1	0.100 ± 0.001	1.00
1	1.01 ± 0.03	2.67
10	9.82 ± 0.18	1.83
1,3-*GDN*		
0.1	0.101 ± 0.002	1.98
1	1.00 ± 0.02	1.80
10	10.0 ± 0.13	1.25

TABLE IV

LINEARITY OF REPRESENTATIVE CALIBRATION CURVES

GTN			1,2-GDN			1,3-GDN		
Spiked concn. (ng/ml)	Peak-height ratio	Calculated concn. (ng/ml)	Spiked concn. (ng/ml)	Peak-height ratio	Calculated concn. (ng/ml)	Spiked concn. (ng/ml)	Peak-height ratio	Calculated concn. (ng/ml)
0.025	0.076	0.026	0.10	0.172	0.103	0.10	0.128	0.110
0.05	0.100	0.047	0.20	0.318	0.207	0.20	0.220	0.187
0.075	0.129	0.073	0.30	0.429	0.287	0.30	0.377	0 318
			0.40	0.591	0.403	0.40	0 481	0.405
0.1	0.172	0.110	0.50	0.731	0.503	0.50	0.553	0.466
0.2	0.262	0 188	0.75	1.064	0.741	0.75	0.904	0.759
0.3	0.397	0.306	1.00	1.435	1.01	1.00	1.197	1.00
Intercept	0.0455			0.0282			−0.0037	
Slope	1.1505			1.3978			1.1956	
r^2	0.9949			0.9994			0.9968	
0.30	0.397	0.310	1.00	1.435	1.05	1.00	1.197	1.06
0.40	0.542	0.427	2.00	2.802	2.01	2 00	2.346	2.01
0.50	0.627	0.495	3.00	4.172	2.96	3.00	3.355	2.85
0.75	0.958	0.762	5.00	7.107	5.01	5.00	5.968	5.02
1.00	1.234	0.984	7 50	10.50	7.38	7.50	9.108	7.62
2.00	2.396	1.92	10.0	14.40	10.1	10.0	11.900	9.94
3.00	3.799	3.05						
Intercept	0.0125			−0.0758			−0.0823	
Slope	1.2409			1.4341			1.2059	
r^2	0.9983			0.9995			0.9992	

were added. The results are listed in Table II. Coefficients of variation for GTN at the concentrations 0.03, 0.1, 1.0 and 10 ng/ml and for 1,2-GDN and 1,3-GDN at the concentrations 0.1, 1.0 and 10 ng/ml were all 5% or less. Calculated concentrations for all samples were within 4% of the actual spiked concentration.

Between-day precision (reproducibility)

The between-day precision was checked by analysis of four concentrations of GTN (0.03, 0.1, 1.0 and 10 ng/ml) and three concentrations of GDNs (0.1, 1.0 and 10 ng/ml) over six days. The results are presented in Table III. The coefficients of variation were $<5\%$ for all concentrations. Calculated concentrations for all samples were within 2% of the actual spiked concentrations.

Linearity

Standard curves of GTN and GDNs were not linear over the entire concentration range of 0.02–10 ng/ml. As described by Noonan et al. [15], this is not due to variation of extraction efficiency since the extraction recovery is the same for low- and high-concentration standards. When standard curves were limited to approximately a ten-fold concentration range, three linear standard curves were obtained for GTN (0.02–0.1, 0.1–1.0 and 1.0–10 ng/ml) and two for the GDNs

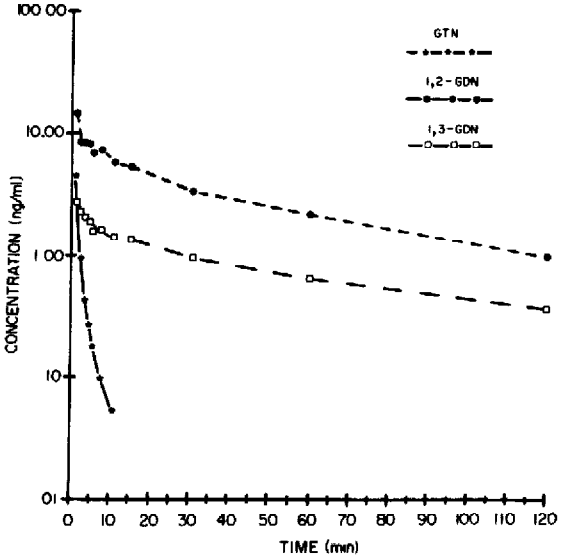

Fig. 2. Venous plasma concentrations of GTN, 1,2-GDN and 1,3-GDN following a single intravenous bolus dose of GTN (0.3 mg) to dog No. 2.

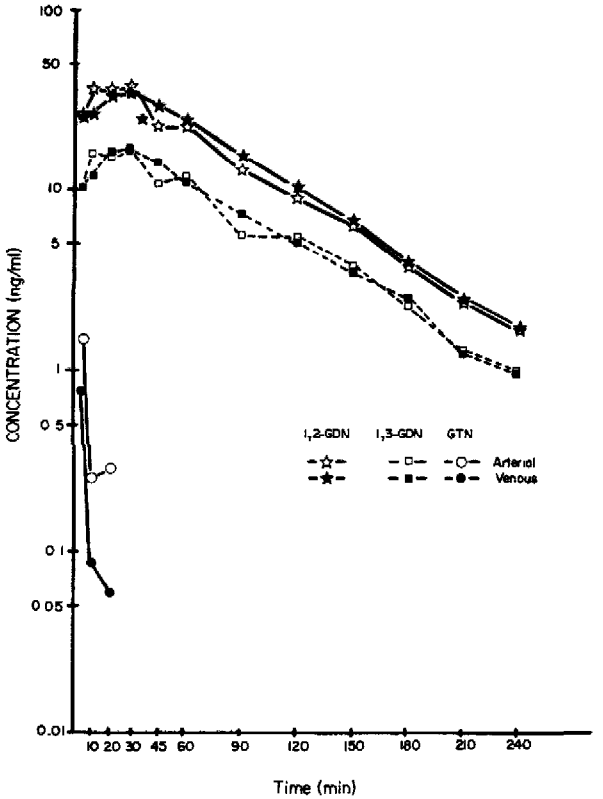

Fig. 3. Arterial and venous plasma concentrations of GTN, 1,2-GDN and 1,3-GDN following a single oral dose of GTN (6.5 mg) to dog No. 2.

(0.1–1.0 and 1.0–10 ng/ml) using weighted linear regression analysis (weight $= 1/y^2$) as demonstrated in Table IV.

Pharmacokinetic studies

This assay method has been used to determine simultaneously the plasma levels of GTN, 1,2-GDN and 1,3-GDN after various dosing routes of GTN in man and dogs. The plasma concentrations versus time plots for parent drug and its dinitrate metabolites following a 0.3-mg intravenous dose and a 6.5-mg oral dose in a dog are shown in Figs. 2 and 3, respectively. The maximum plasma concentrations for GTN, 1,2-GDN and 1,3-GDN following the intravenous dose were 4.39, 14.4 and 2.70 ng/ml, respectively; following the oral dose they were 0.78, 34.7 and 17.1 ng/ml, respectively. The half-lives determined for GTN, 1,2-GDN and 1,3-GDN after the intravenous bolus dose were 2.5, 52 and 68 min, respectively. These values are very close to those reported previously in dogs [13] and man [1,14,16–18].

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REFERENCES

1 P.W. Armstrong, J.A. Armstrong and G.S. Marks, Circulation, 66 (1982) 1273.
2 P.A. Cossum, M.S. Roberts, A.J. Galbraith and G.W. Boyd, Lancet, ii (1978) 349.
3 J.A. Armstrong, S.E. Slaughter, G.S. Marks and P.W. Armstrong, Can. J. Physiol. Pharmacol., 58 (1980) 459.
4 P.K. Noonan and L.Z. Benet, Int. J. Pharm., 12 (1982) 331.
5 M.T. Rosseel and M.G. Bogaert, J. Pharm. Sci., 62 (1973) 754.
6 P.S.-K. Yap, E.F. McNiff and H.-L. Fung, J. Pharm. Sci., 67 (1978) 582.
7 C.C. Wu, T.D. Sokoloski, A.M. Burkman, M.F. Blanford and L.S. Wu, J. Chromatogr., 228 (1982) 333.
8 A. Sioufi and F. Pommier, J. Chromatogr., 339 (1985) 117.
9 A. Sioufi, F. Pommier and J.P. Dubois, J. Chromatogr., 413 (1987) 101.
10 R.J. Spanggord and R.G. Keck, J. Pharm. Sci., 69 (1980) 444.
11 P. Ottoila, J. Taskinen and A. Sothmann, Biomed. Mass Spectrom , 9 (1982) 108.
12 A. Gerardin, D. Gaudry and D. Wantiez, Biomed. Mass Spectrom., 9 (1982) 333.
13 H. Miyazaki, M. Ishibaski, Y. Hashimoto, G. Idzu and Y. Furuta, J. Chromatogr., 239 (1982) 277.
14 J.A. Settlage, W. Gielsdorf and H. Jaeger, J. High Resolut. Chromatogr. Chromatogr. Commun., 6 (1983) 68.
15 P.K. Noonan, I. Kanfer, S. Riegelman and L.Z. Benet, J. Pharm. Sci., 73 (1984) 923.
16 P.K. Noonan, R.L. Williams and L.Z. Benet, J. Pharmacokinet. Biopharm., 13 (1985) 143.
17 M.G. Bogaert and M.T. Rosseel, J. Pharm. Pharmacol., 24 (1972) 737.
18 A. Bashir, M.J. Lewis and A.H. Henderson, Br. J. Clin. Pharmacol., 14 (1982) 779.