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Determination of nitroglycerin and its dinitrate metabolites in urine by gas chromatography–mass spectrometry as potential biomarkers for occupational exposure

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Abstract

This paper describes a method for the quantitative analysis of nitroglycerin and its dinitrate metabolites (1,2- and 1,3-glycerol dinitrate) in urine. After liquid–liquid extraction the analytes were separated and quantified using gas chromatography–mass spectrometry with negative ion chemical ionisation. The method can detect above 0.3 nmol/l for all analytes and is linear over the range of at least 0–44 nmol/l. The method was then used to determine metabolite levels in a subject using nitroglycerin therapeutically for the treatment of angina. Metabolites were stable in urine for at least 6 days at room temperature, ~4°C and −20°C.

Keywords: Nitroglycerin; Glycerol dinitrate

1. Introduction

Nitroglycerin or glycerol trinitrate (GTN) has uses both in the explosives and pharmaceutical industries. Occupational exposure to GTN may occur during the manufacture and use of dynamite and some rocket propellants. GTN is readily absorbed through the skin and is a powerful vasodilator, and a commonly used anti-anginal agent. Occupational exposure to GTN can result in headaches and dizziness and because of its effects on heart muscle there are concerns for those occupationally exposed, both during periods of exposure and periods away from exposure.

The UK occupational exposure standard (OES) for an 8 h period for GTN is set at 0.2 ppm (1.9 mg/m^3). The short term exposure limit (15 min) is also 0.2 ppm. GTN is readily absorbed through the skin and it has been given an ''Sk'' notation [1]. The Deutsche Forschungsgemeinschaft have set biological monitoring tolerance values for GTN exposure at 0.5 μg/l 1,2- and 1,3-glycerol dinitrate (GDN) in plasma [2].

The metabolism of GTN occurs via denitration of the molecule to initially form the 1,2- and 1,3-glycerol dinitrates. It is thought that this occurs with glutathione [3]. Following administration of radiolabelled GTN to rats [4] 2% was excreted as 1,3-GDN, 6% as 1,2-GDN. Thirty-three percent of the GTN was excreted as glycerol mononitrate (GMN). Eight percent of the GTN was excreted as glycerol, and between 17 and 33% as CO_2. The ratio of 1,2-GDN to 1,3-GDN concentrations found in human plasma appear to vary depending on the route of administration [5]. The ratios are 7:1 following intravenous administration; 4:1 after dermal applica-

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tion, and 2:1 after oral administration. The half-life of GTN in man is between 1 and 3 min [6] and for both dinitrate metabolites of GTN in human plasma is between 30 and 50 min [7,8].

Because of the strong possibility of dermal absorption, biological monitoring of nitroglycols would be a useful aid in assessing occupational exposure. Methods for measuring levels of nitroglycols in blood are available, but the short half-lives [7] make sampling time critical and the measurements can only give an estimate of very recent exposure. These difficulties have so far limited the application of biological monitoring for GTN. However, a biological monitoring strategy based on urine sampling offered a way forward, if a suitable method could be developed.

Most of the literature on GTN metabolism and analysis is concentrated on methods involving plasma [8–17] therefore in order to carry out an assessment of the feasibility of urine sampling as a means of nitroglycerin exposure assessment the development of a urine method was required. The development and evaluation of such a method is described here and involves the analysis of GTN, 1,2- and 1,3-GDN in urine.

2. Experimental

2.1. Conditions

2.1.1. Chemicals and reagents

The two metabolites 1,2- and 1,3-glycerol dinitrate solutions (1 mg/ml) were from Promochem (Welwyn Garden City, UK), and glycerol trinitrate solution (10 μg/ml) was obtained from Qm_x Labs. (Thaxted, UK). Methanol and toluene (HPLC grade) were from Rathburn (Walkerburn, UK) and diisopropyl ether and silver nitrate (AR grade) were from Fisher Scientific (Loughborough, UK). The internal standard *p*-nitrobenzyl alcohol (99%) and dichlorodimethylsilane (99%) were from Sigma–Aldrich. Double distilled water was prepared in the laboratory using a Milli-Q Plus ultra pure water system.

2.1.2. Equipment

A Hewlett-Packard Model 5890 Series 2 gas chromatograph with automated injection (Hewlett-Packard, Cheshire, UK) was used attached to a Trio-1 Mass Spectrometric Detector with Masslab software (VG Masslab, Altrincham, UK). A 30 m× 0.32 mm ZB-1 capillary column with 1 μm film thickness (Phenomenex, Macclesfield, UK) was fitted. Helium was used as a carrier gas with a column head pressure of 35 kPa. The injection port temperature was 150°C, and the column oven temperature was initially 110°C rising by 5°C/min to 124°C, then by 10°C/min to 140°C, then by 30°C/min to 180°C remaining there for 2 min. The GC–MS transfer line was at 280°C. The mass spectrometer was operating in the negative chemical ionisation mode with the source at 200°C and the quad at 100°C. The reagent gas used for chemical ionisation was methane. Detection was in the selected ion monitoring (SIM) mode with a solvent delay of 1.2 min. The ions monitored (with dwell time of 80 ms) were m/z 46 and 62 for 1,2- and 1,3-GDN and GTN and m/z 152 for *p*-nitrobenzyl alcohol. Centrifugation was carried out on a MSE Mistral 2000 centrifuge (Fisher Scientific).

2.1.3. Sample preparation

Prior to any extraction all glassware used was silanised by placing in a 5% solution of dichlorodimethylsilane in toluene for 30 min, then rinsing in toluene before heating them at 110°C for 1 h.

Calibration samples were prepared for each analysis run by adding appropriate volumes of 1,2- and 1,3-GDN and GTN analyte stock solutions to 2 ml of urine obtained from an individual not exposed to GTN or GDN in polypropylene tubes. Sample and control urine (2 ml) were also added to polypropylene tubes. A 100-μl volume of 10 μg/ml *p*-nitrobenzyl alcohol in methanol was added to each calibrator, sample and control followed by 100 μl of 20 m*M* $AgNO_3$ solution and 4 ml of diisopropyl ether. The capped tubes were then tumble mixed for 20 min, before centrifugation at 1614 *g* for 10 min. The ether layer was then transferred to a second tube and evaporated to dryness under a stream of nitrogen. The analytes were then reconstituted in 100 μl of toluene which was transferred into GC vials, from which 2 μl was injected into the GC system.

2.2. Initial evaluation

Urine from a subject not exposed to nitroglycols, urine spiked with GTN, 1,2-GDN and 1,3-GDN and

a sample of urine from an individual taking GTN for therapeutic purposes were analysed to ascertain the relevant chromatographic peaks of the compounds of interest and to investigate the levels that might be detected in urine from an exposed individual.

2.3. Linearity

Calibration samples to assess the linearity of the method were prepared for all three analytes (GTN, 1,2- and 1,3-GDN). A working standard solution containing each analyte was prepared in methanol (conc. 440 nmol/l GTN and 550 nmol/l 1,2- and 1,3-GDN). This solution was then used to prepare six calibration standards in urine over the ranges 0–44 nmol/l GTN and 0–55 nmol/l 1,2- and 1,3-GDN. An internal standard (I.S.), *p*-nitrobenzyl alcohol was added to each standard. Following extraction and analysis linearity was assessed by a least-squares linear regression of the analyte to I.S. peak height ratios against urine concentrations. This was performed daily on 5 consecutive days.

2.4. Detection limit

The detection limit for the three analytes in urine was investigated. Samples with concentrations of 0.44, 0.22, 0.04 and 0 nmol/l GTN and 0.55, 0.28, 0.06 and 0 nmol/l 1,2- and 1,3-GDN were analysed. The detection limit was defined as that level which could be detected at three times the background noise.

2.5. Sample stability

A spiked sample was prepared at a concentration of 22 nmol/l GTN and 27.5 nmol/l 1,2- and 1,3-GDN by adding stock solution (conc. 440 nmol/l GTN and 550 nmol/l 1,2- and 1,3-GDN) to urine from an unexposed individual. A sample of urine collected from an individual using GTN therapeutically was also prepared. Aliquots of these two solutions were placed in universal urine containers and stored under three different conditions: (i) room temperature out of direct sunlight; (ii) refrigeration in the dark at ~4°C; and (iii) frozen at ~−20°C. Aliquots were analysed with freshly prepared calibration standards on days 0, 2, 5, and 6 following preparation, where day 0 was the day of preparation.

2.6. Reproducibility

Quality control material was prepared using the urine of an unexposed individual which was spiked at a concentration of 13.2 nmol/l GTN and 16.5 nmol/l 1,2- and 1,3-GDN. Aliquots were frozen at ~−20°C and used to assess intra- and inter-assay variation. Intra-assay variation was determined using 10 samples from the same analysis run, and the inter-assay variation was determined using 10 analysis runs. The reproducibility of the instrumentation was also calculated by injecting the same sample 10 times in succession.

2.7. Human study

A urine sample was collected from an individual using GTN therapeutically on 4 separate days. The samples were collected between 60 and 95 min following sublingual dosing with approximately 0.4 mg of GTN. They were analysed to determine the urine levels of GTN, and its metabolites 1,2- and 1,3-GDN.

2.8. Results and discussion

2.8.1. Initial evaluation

The spiked samples exhibited chromatographic peaks for 1,3- and 1,2-GDN and GTN for SIM at m/z 62, and m/z 46 with retention times of 4.4, 4.6, and 4.9 min, respectively, and internal standard peak for SIM at m/z 152 with a retention time of 6.6 min. No such peaks were evident in the unexposed urine (see Fig. 1). The four samples collected from an individual therapeutically exposed to GTN exhibited peaks corresponding to 1,2- and 1,3-GDN, but no GTN peak. The GDN peak heights were within the range of the standard curve for this study.

2.8.2. Linearity

The method is linear over the range of 0–44 nmol/l for GTN and 0–55 nmol/l for 1,2- and 1,3-GDN. Table 1 gives a summary of the linearity regression data. The top of the linearity range appears to be sufficiently high to analyse samples

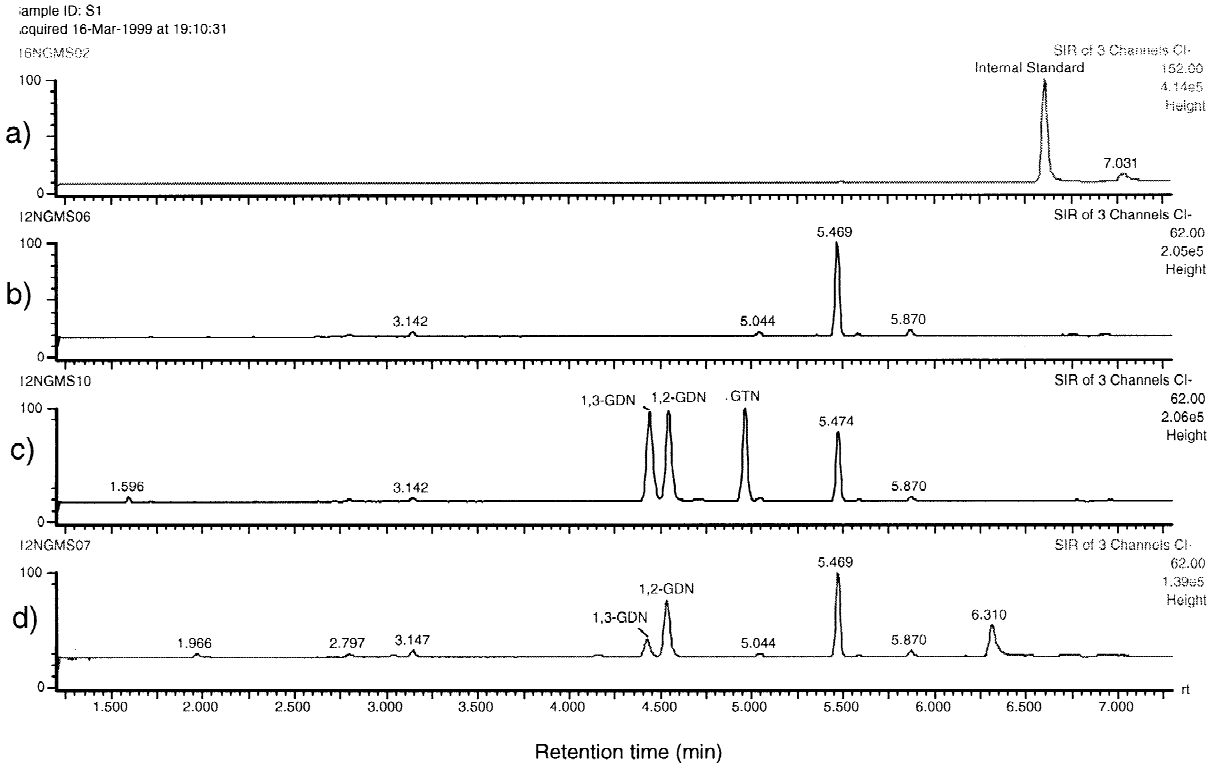

Fig. 1. Single ion chromatograms of (a) a sample of urine from an unexposed individual spiked with internal standard (m/z 152); (b) a sample of unexposed urine (m/z 62); (c) a urine sample spiked with 1,3- and 1,2-GDN (55 nmol/l) and GTN (44 nmol/l) (m/z 62); and (d) a sample from an individual dosed with GTN, containing 1,3-GDN (3.6 nmol/l) and 1,2-GDN (12.4 nmol/l) (m/z 62). Chromatograms are normalised to show the highest peak at 100%.

from therapeutically exposed individuals, however a study of occupationally exposed individuals would be needed to ascertain if the range is sufficient for biological monitoring of occupational exposure.

2.8.3. Detection limit

No chromatographic peak was visible for the sample spiked at 0.04 nmol/l GTN and 0.06 nmol/l 1,2- and 1,3-GDN. However the sample spiked at 0.22 nmol/l GTN and 0.28 nmol/l 1,2- and 1,3-GDN exhibited peaks at more than three times the background noise. Therefore the detection limits were set at 0.22 nmol/l for GTN and 0.28 nmol/l for 1,2- and 1,3-GDN. These levels are well below those detected in urine from an individual using GTN therapeutically, however evaluation of urine collected following occupational exposure would reveal

Table 1
Regression data of calibration curves used for linearity assessment

Analyte	Slope (SD)	Intercept (SD)	Correlation coefficient
1,2-GDN	0.0327 (0.0092)	−0.0117 (0.0145)	0.9966
1,3-GDN	0.0303 (0.0082)	−0.0042 (0.0199)	0.9982
GTN	0.0279 (0.0069)	0.0019 (0.0373)	0.9963

SD=Standard deviation.

Table 2
Intra- and inter-assay relative standard deviations and system reproducibility

	Intra-assay (n=10)			Inter-assay (n=10)			System reproducibility (n=10)		
	1,2-GDN	1,3-GDN	GTN	1,2-GDN	1,3-GDN	GTN	1,2-GDN	1,3-GDN	GTN
x (nM)	14.2	16.6	12.0	15.4	17.6	12.7	17.2	20.1	14.3
SD (nM)	1.2	1.6	1.3	1.8	1.9	1.5	0.5	0.6	0.5
RSD (%)	8.1	9.5	11.2	11.5	11.0	12.1	3.1	2.9	3.8

x=Mean concentration, SD=standard deviation, RSD=relative standard deviation.

if these levels are sufficiently low for monitoring occupational exposure.

2.8.4. Sample stability

The stability of the spiked sample and the sample from an exposed individual was maintained over at least 6 days at ~4°C (refrigerated) and at ~−20°C (frozen). At room temperature, however, degradation of 1,2- and 1,3-GDN in the spiked samples occurred after only 2 days, although this was not the case for the samples from the exposed individual which remained stable over at least 6 days. The GTN in the spiked sample stored at room temperatures showed almost immediate instability and was not even stable for 2 days. The difference in stability between the spiked samples and the samples from the exposed individual may be due to different sample pH or sterility, which were not measured. These results show that following collection, samples from individuals occupationally exposed to GTN can be sent to the laboratory for analysis by first-class post without any concerns relating to significant degradation of the metabolite levels.

2.8.5. Reproducibility

Table 2 gives a summary of the intra- and inter-assay relative standard deviations and system reproducibility. These results are sufficient for setting good internal quality control limits for this method.

2.8.6. Human study

Although no GTN was detected in the four samples collected from an individual therapeutically exposed to GTN, metabolites were detected, and the concentration ranges were 10.45–16.50 nmol/l 1,2-GDN and 2.81–5.17 nmol/l 1,3-GDN. The mean ratio of 1,2-GDN concentration to 1,3-GDN concentration in these samples was 3:1.

3. Conclusion

Very little data exist on the elimination of GTN and metabolites from urine. Plasma half-lives following oral administration of GTN are 1–3 min for GTN [6] and 30–50 min for the metabolites 1,2- and 1,3-GDN [7,8]. If significant proportions of the dinitrate metabolites are excreted via the urine then, at these plasma clearance rates, the analysis of the urine may provide a convenient method for monitoring GTN exposure provided a reliable analytical method could be identified.

The results obtained from this study would suggest that this method is both sensitive and reproducible and with a sufficiently large range to offer a realistic tool for the assessment of therapeutic exposure.

It is not yet clear how occupational exposures compare with therapeutic levels and further work using samples from subjects occupationally exposed to GTN, therefore, is clearly required to confirm whether the method's linearity range and detection limits would be sufficiently adequate to identify and measure dinitrate metabolites after occupational exposure. Only then, in the light of positive results, could a sampling strategy for occupational exposure assessment using biological monitoring be defined.

Acknowledgements

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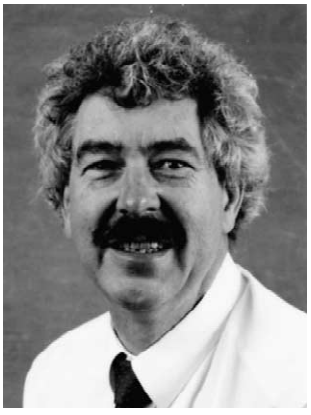
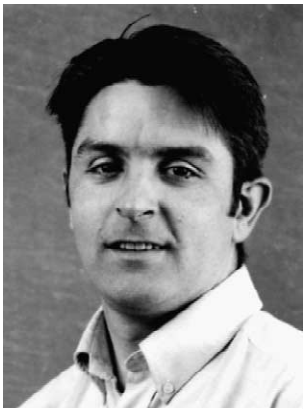
References

[1] EH40/2000 Occupational Exposure Limits 2000, HSE Books, Sudbury, 2000.
[2] List of MAK and BAT Values, 1999 Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area, Report No. 35, Wiley–VCH, Weinheim, 1999.
[3] P. Needleman, J.C. Krantz Jr., Biochem. Pharmacol. 14 (1965) 1255.
[4] P. Needleman, Annu. Rev. Pharmacol. Toxicol. 16 (1976) 81.
[5] P.K. Noonan, L.Z. Benet, Clin. Pharmacol. Ther. 42 (1987) 273.
[6] M.G. Bogaert, M.T. Rosseel, J. Pharm. Pharmacol. 24 (1972) 737.
[7] D.K. Yu, R.L. Williams, L.Z. Benet, E.T. Lin, D.H. Giesing, Biopharm. Drug Dispos. 9 (1988) 557.
[8] H.M. Bolt, in: H. Greim (Ed.), Biological Exposure Values for Occupational Toxicants and Carcinogens: Critical Evaluation for BAT and EKA Values, Vol. 3, Wiley–VCH, Weinheim, 1998, p. 143.
[9] A. Gerardin, D. Gaudry, D. Wantiez, Biomed. Mass Spectrom. 9 (1982) 333.
[10] P. Ottoila, J. Taskinen, A. Sothmann, Biomed. Mass Spectrom. 9 (1982) 108.
[11] A. Sioufi, F. Pommier, J.P. Dubois, J. Chromatogr. B 413 (1987) 101.
[12] J.J. Janssens, M.I. Selala, F.F. Daelmans, S.W. Andries, P.J.C. Schepens, J. Pharm. Biomed. Anal. 7 (1989) 1631.
[13] B.P. Booth, B.M. Bennett, J.F. Brien, D.A. Elliott, G.S. Marks, J.L. McCans, K. Nakatsu, Biopharm. Drug Dispos. 11 (1990) 663.
[14] K. Torfgard, J. Alhner, B. Norlander, J. Chromatogr. B 534 (1990) 196.
[15] C. Han, M. Gumbleton, D.T.W. Lau, L.Z. Benet, J. Chromatogr. B 579 (1992) 237.
[16] M. Jorgensen, M.P. Andersen, S.H. Hansen, J. Chromatogr. B 577 (1992) 167.
[17] A. Khan-Raja, P.R. Robinson, K.J. Barrell, J. Maddock, Methodol. Surv. Bioanal. Drugs 25 (1998) 86.



John Cocker is an analytical biochemist with extensive experience in developing and using sophisticated techniques to analyse biological samples for trace levels of toxic substances or their metabolites. He is head of a team of 6–8 graduate and postdoctoral scientists in the Organic Toxicology Section of the Health and Safety Laboratory, an Agency of the UK Health and Safety Executive. The section provides a biological monitoring service to HSE and private firms to help with assessment of occupational exposure to hazardous substances in general and in particular, those which may be absorbed through the skin or where control of exposure relies on respiratory protection. The Section also conducts research and development, including human volunteer studies, to provide new methods and data to aid the interpretation of results. Dr. Cocker has over 20 years experience in biological monitoring and is an advisor within HSE, to private firms and to the World Health Organisation on biological monitoring methods, policy, setting guidance values and interpreting results.



Peter Akrill was born in 1971 in Cardiff (UK). He studied Chemistry at the University of Wales College, Cardiff and graduated with honours in 1993. He then worked for 2 years for Cardiff Bioanalytical Services in therapeutic drug monitoring and chemical analysis for clinical trials. In 1996 he moved to the Organic Toxicology section of the Health and Safety Laboratory, where he has particular involvement in the development of biological monitoring methods for the assessment of workplace exposure to chemicals.