Journal of Pharmaceutical & Biomedical Analysis
Vol. 5, No. 3, pp. 275–282, 1987
Printed in Great Britain

0731–7085/87 $3.00 + 0.00

Analysis of solutions containing glutathione and inorganic nitrite: application to nitroglycerin metabolism studies

STEPHEN H. CURRY[1]*, SUHER M. ABURAWI[1] and ROBIN WHELPTON[2]

[1] *Division of Clinical Pharmacokinetics, College of Pharmacy, J. Hillis Miller Health Center, University of Florida, Gainesville, FL 32610, USA*
[2] *Department of Pharmacology and Therapeutics, The London Hospital Medical College, Turner Street, London E1 2AD, UK*

Abstract: Nitroglycerin (GTN) is metabolized to 1,2-dinitroglycerin (1,2-GDN) and 1,3-dinitroglycerin (1,3-GDN) *in vivo* and in liver homogenates. 1,2-GDN and 1,3-GDN are converted to isomers of glyceryl mononitrate (GMN) *in vivo*. The denitration reactions yield inorganic nitrite (NO_2^-) which is oxidized to inorganic nitrate (NO_3^-). Denitration involves utilization of glutathione (GSH). In attempting to use the Bratton–Marshall assay for NO_2^- in studies of GTN metabolism *in vitro*, and in attempting to use Ellman's reagent for GSH in the same research, apparent concentrations of both NO_2^- and GSH were noticed lower than anticipated. Apparent mutual interference by NO_2^- and GSH in their respective assays was then found. Development of a specific liquid chromatographic method for measurement of NO_2^-, NO_3^-, GSH and oxidized glutathione (GSSG) permitted the study of the interaction of NO_2^- and GSH, which yielded NO_3^- and GSSG.

Keywords: *Nitroglycerin; glutathione; nitrate; nitrite; Bratton–Marshall assay; Ellman's reagent; high-performance liquid chromatography.*

Introduction

Glyceryl trinitrate (GTN) is metabolized to a group of partially denitrated analogues (1,2-glyceryldinitrate, 1,3-glyceryldinitrate, 1-glycerylmononitrate and 2-glycerylmononitrate), and to nitrite and nitrate ions [1–8]. The same products result from chemical decomposition, but the mechanisms involved are different [9]. Metabolic denitration involves the enzyme, organic nitrate reductase and reduced glutathione. This reaction is explained as a reduction of GTN to a nitrite ester, followed by hydrolysis. The product of metabolic denitration is inorganic nitrite, which is then converted to inorganic nitrate.

Metabolic studies with GTN *in vitro* require assay methods for GTN and its products individually in the presence of each other, and also for the reduced (GSH) and oxidized (GSSG) forms of glutathione. Assay of glutathione in the presence of GTN and of its metabolites is desirable. In this report, results of assay development experiments are

* To whom correspondence should be addressed.

described which disclose that a chemical reaction between nitrite, nitrate, GSH and GSSG can occur.

Materials and Methods

Materials

GTN was obtained as a 10% adsorbate on lactose (as a gift from ICI Americas Inc., Wilmington, Delaware, USA) and as a 10% solution in ethanol (as a gift from Arthur H. Cox Ltd., Brighton, UK). Samples of 25% lactose adsorbate of isosorbide dinitrate (ISDN) were obtained from Ayerst Laboratories, Farnborough, UK and from ICI Americas. Stock solutions of GTN were prepared at a concentration of 0.1 mg/ml of GTN in water by dissolving the adsorbate or by dilution of the ethanolic solution. Potassium nitrate, sodium nitrite and other reagents were analytical grade.

Preparation of a GTN lactose adsorbate sample

Where the lactose adsorbate was unavailable, it was prepared as follows in order to avoid the presence of ethanol in biological experiments. A 1 ml sample of the 10% ethanolic solution was placed in a glass vial, and then 1.9 g of lactose and 1 ml of ethanol were added. The mixture was stirred, and then the ethanol was gently evaporated under a stream of nitrogen with occasional stirring. When the GTN lactose mixture was free moving as a powder it was weighed. The vial was then placed in a vacuum pistol and heated with refluxing methylene chloride (B.P. 38–41°C). The sample was dried to constant weight.

Standardization of GTN lactose adsorbates and ethanolic solutions

Samples of both types of material were standardized by the method of the British Pharmacopoeia [10] in which NO_3^- ions are quantitatively removed from the compound to be assayed by hydrolysis and used to nitrate phenoldisulphonic acid. Standardization is by comparison with potassium nitrate. This method was used without modification except for minor changes in the volumes of samples.

Synthesis of partially nitrated glycerols

1,2-Glyceryl dinitrate (1,2-GDN) and 1,3-glyceryl dinitrate (1,3-GND) were synthesized by refluxing 2,3-dibromo-1-propanol and 1,3-dibromo-1-propanol, respectively with silver nitrate. Glyceryl mononitrate was synthesized by partial nitration of glycerol. The reaction products were extracted from the reaction mixtures into ether. The ether extracts were dried over anhydrous magnesium sulphate and the ether was removed under reduced pressure. The residues were redissolved in ethanol and the products were purified by thin layer chromatography.

Thin layer chromatography (TLC)

Glass-backed silica gel TLC plates with fluorescent indicator incorporated (20 × 20 cm, 0.25 mm thick, Kodak) were used. On each plate a pencil base line was drawn, to mark the origin, 1 cm from the bottom edge of the plate, and two vertical lines were drawn 1.5 cm from the side edges of the plates. Two drops of the ethanol extracts, prepared as described in the previous section, were placed at the two ends of the base line and in each case a separate band of the extract was spotted inside the middle 17 cm of the base line. The elution system was either ethyl acetate–*n*-heptane (9:1, v/v) [4] for

the identification and separation of GMN, or benzene–ethyl acetate (4:1, v/v) [5] for the identification and separation of 1,2-GDN and 1,3-GDN. After development, the middle part of the plate was masked with card and the two sides sprayed with 1% ethanolic diphenylamine [5]. The plate was also examined by exposure to ultraviolet light. The zones containing the required products were scraped from the plates and the compounds eluted with ethanol. The ethanolic solutions were standardized as described earlier for GTN, with appropriate corrections for the nitrate content of the compounds.

Preparation of liver homogenates

The soluble fraction of liver homogenate was prepared as described previously [6]. The liver was removed from male Sprague–Dawley or Wistar rats (wt. range 150–200 g), washed with 0.0673 M phosphate buffer (pH 7.4) containing 0.25025 M sucrose, and then homogenized with 3 volumes of a fresh sample of the same solution to give a 25% w/w homogenate. The homogenate was centrifuged at 9,000 **g** (10,000 rpm) at 5°C for 25 min. The supernatant was decanted and used.

Metabolic incubation of GTN

The usual incubation mixture was initially one part liver homogenate and one part phosphate buffer 0.067 M (pH 7.4) with GSH added to give 2.5 mg/ml (8.14 μmole/ml). This mixture was then pre-incubated at 37°C for 1 h. The appropriate quantity of GTN dissolved in a small volume of water (0.1 ml) was then added and incubation was continued in a Dubnoff shaking incubator for up to 1 h. Samples of the mixture (0.1 ml) were drawn at chosen time intervals and added to a previously prepared solution of isosorbide dinitrate in methanol. The mixtures were shaken and centrifuged, and the supernatants were assayed by various methods. The methanol stopped metabolic activity, and the isosorbide dinitrate was present to serve as an internal standard in liquid chromatographic assays (see later).

Measurement of NO_2^- *by the Bratton–Marshall assay*

The method of Bratton and Marshall [11, 12] was modified for the determination of NO_2^-. Samples of the incubation mixture (0.1 ml) were added to samples (0.2 ml) of a solution of 5% mercuric chloride [5]. The mixture was centrifuged. A sample of the supernatant (0.1 ml) was added to 2 ml of 5% trichloroacetic acid, and then 1 ml of a solution of sulphanilamide (0.2%) was added. The mixture was left standing for 10 min. One ml of a solution (0.1%) of *N*-1-naphthyl-ethylene diamine (NED) was then added and the mixture was again allowed to stand for 10 min, during which colour developed. The colour was assessed in a colorimeter at 540 nm using a trichloroacetic acid blank, and standard solutions of inorganic nitrate in blank liver homogenate.

Measurement of glutathione with Ellman's reagent

One ml samples of the supernatant layers from 25% w/w liver homogenates were added to 4 ml of a solution containing 5% trichloroacetic acid and 5 mM/l ethylenediaminetetraacetic acid. The mixture was centrifuged. Samples (0.1–1.0 ml) were diluted to 9.9 ml in duplicate with 0.1 M phosphate buffer (pH 8.2), and added to 0.1 ml samples of Ellman's reagent, 5,5′-dithiobis-(2-nitrobenzoic acid), 3.96 mg/ml in methanol. The mixture was shaken and left for 20 min at room temperature. The optical density of the yellow colour that developed was measured at 410 nm against a reagent blank prepared in the same way but with the substitution of 0.1 ml of 0.1 M phosphate

buffer (pH 8.2) for Ellman's reagent. The reduced glutathione concentration was calculated by comparison with a standard curve generated from solutions of known concentration of glutathione in place of the liver homogenate sample [13].

Liquid chromatography of organic nitrites

A 300 × 4 mm i.d. column packed with 10-μm Spherisorb ODS was employed with a pre-column of silica (Co-pell ODS). The mobile phase was HPLC grade methanol mixed with double distilled water, and passed through a Whatman glass microfibre filter, 0.7 μm, (GF/F). The solvent was degassed for 5 min in an ultrasonic shaker before use. The methanol concentration was optimally 50% v/v for identification and quantitation of GTN [14], but reducing the concentration to 40% allowed the measurement of GTN and the isomers of GDN and GMN in the same solution. The flow rate was 1 ml/min. Isosorbide dinitrate was used at a concentration of 25 μg/ml as an internal standard and quantitation was by means of peak height ratios. GTN and its nitric acid ester metabolites were measured in 0.1 ml volumes of liver homogenates (see earlier) by comparison with standards prepared in methanol. A control experiment had earlier shown that the calibration graphs for GTN in methanol and in liver homogenates were not significantly different in slope or intercept. The apparatus consisted of either: (i) a Waters M-6000A pump, a Waters U6K injection port, a Jasco Uvidec-100-III variable wavelength UV detector and a Varian Multivolt Model 9176 recorder, or (ii) an LDC Constametric III complete system. The detection wavelength was 210 nm.

Liquid chromatography of NO_2^-, NO_3^-, *GSH and GSSG in admixture*

A C_{18}-microBondapak column was used with a mobile phase of 0.1 M disodium hydrogen phosphate and 0.1 N HCl in proportions to give a pH of 2.0. The flow rate was 2 ml/min and detection was by ultraviolet spectrophotometry at 210 nm. Prior to analysis by liquid chromatography, samples of liver homogenate were deproteinated with 1% picric acid in water (4 ml per 1 ml of homogenate). The mixture was centrifuged and samples (20 μl) were injected into the chromatograph. Quantitation was by comparison with aqueous solutions of the compounds treated as described for liver homogenates.

Results and Discussion

The GTN lactose adsorbate obtained from ICI Americas contained 9.09 ± 0.08% GTN (mean ± S.E.M.). Lactose adsorbate prepared in our own laboratories contained 4.96 ± 0.135% GTN (mean ± S.E.M., $n = 5$). In each case, the appropriate quantity of lactose adsorbate was weighed to give GTN concentrations in solutions exactly as required. The ethanolic GTN solution contained 9.42 ± 0.58% GTN (mean ± S.E.M., $n = 5$).

The quantitative methods used all gave linear calibration graphs and were used in concentration ranges at least 10 times the minimum detectable quantity. For example, the linear response to GSH measured by liquid chromatography ranged from at least 95 ng/ml (for GSH) to 1 mg/ml, and concentrations of GSH measured in liver homogenates were in the range 0.3–0.4 mg/ml. Coefficients of variation, both between-day and within-day were always less than 10%. Calibration graphs with each batch of assay were routinely set up.

The question arose as to whether lactose and/or ethanol would affect GTN metabolism. Pilot experiments showed that at high ethanol concentrations inhibition of

GTN metabolism did indeed occur. There was no similar effect with lactose. The ethanol problem was overcome by preparing lactose adsorbates from ethanol solutions and thus eliminating ethanol from all incubations.

GTN was converted to 1,2-GDN and 1,3-GDN when incubated in 25% w/w liver homogenates fortified with GSH. Figure 1 shows the disappearance of GTN and formation of the two products in a typical experiment. Inorganic nitrite (by the Bratton–Marshall assay) and both inorganic nitrite and inorganic nitrate (by LC assay) were also detected as products. The disappearance of GTN occurred rapidly at first and then slowly. Typically, with a starting concentration of GTN of 1 mg/ml, the GTN concentration reached less than 0.6 mg/ml within 5 min, while the GSH concentration fell even more dramatically from 2.5 mg/ml to a mean of 0.028 mg/ml (S.D. 0.0016, n = 5) or by almost 99%. These incubations provided evidence that reactions occurred and confirmed observations of others so that no further investigation was required.

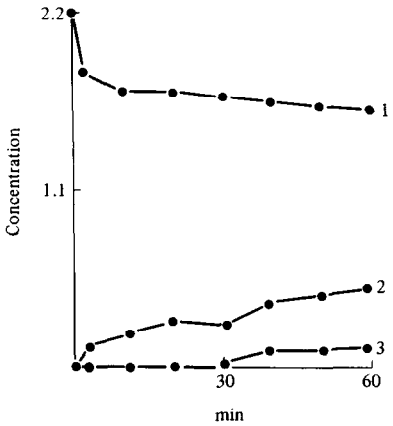

Figure 1
Disappearance of nitroglycerin (1) and appearance of 1,2-dinitroglycerin (2) and 1,3-dinitroglycerin, when incubated in a liver homogenate at a starting concentration of 22 μM/ml.

Although nitrite ions were detected as present by the Bratton–Marshall assay for nitrite, the apparent concentrations recorded were much less than predicted on the basis of production of one mole of nitrite per denitration reaction, even allowing for partial conversion to nitrate. This led to the suspicion that GSH might be interfering with the nitrite assay, which had been validated for liver homogenates and aqueous solutions without added GSH.

When calibration graphs for this assay were generated with and without added GSH, the results shown in Fig. 2 were obtained. These results show interference by GSH in the Bratton–Marshall assay of nitrite suggesting that there might be a reaction between GSH and nitrite. If this were so, there should be an analogous interference by nitrite in GSH assays using Ellman's reagent. We had earlier found that the GSH concentration in the liver of nitrite-free rats was 1.22 ± 0.04 (mean ± S.E.M.) mg/g. When calibration graphs for GSH using Ellman's reagent were generated with and without added nitrite, the results shown in Fig. 3 were obtained. These results show interference by nitrite in the assay of GSH, reinforcing the suggestion that there might be a reaction between GSH and nitrite.

The occurrence of a reaction between GSH and nitrite was first reported in 1970 [8], when the use of acetic anhydride to eliminate the influence of GSH on NO_2^- assays was described. We decided to study this reaction using liquid chromatography. When a

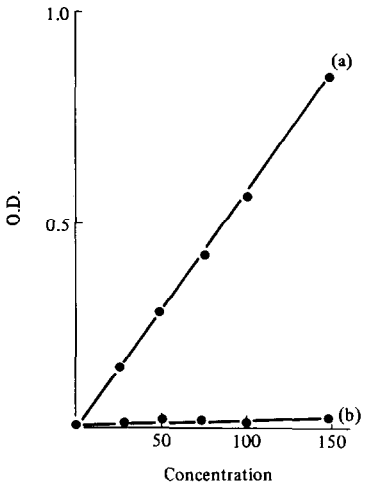

Figure 2
Calibration graph for Bratton–Marshall assay of nitrite over the range 0–150 μg/ml: (A) without glutathione; (B) with glutathione (1 mg/ml).

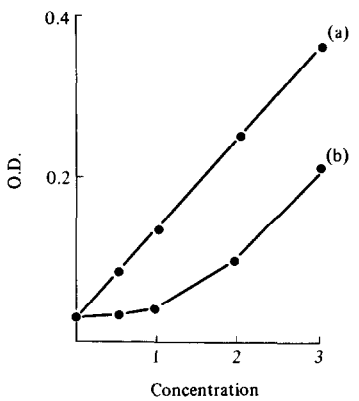

Figure 3
Calibration graph for the assay of glutathione using Ellman's reagent over the range 0–3 mg/ml: (A) without nitrite; (B) with nitrite (150 μg/ml).

solution of GSH and sodium nitrite (0.5 mg/ml of each) was prepared in water, and subjected to liquid chromatography, it initially showed the presence of only GSH and NO_2^- ions. After 15 min at 25°C, small signals of oxidized glutathione (GSSG) and nitrate ions were detectable. After 210 min, there was a substantial reduction in the GSH and NO_2^- peaks with clear evidence of GSSG and NO_3^- production, indicating the occurrence of the following reaction (Figs 4 and 5):

$$2GSH + NO_2^- \rightleftharpoons GSSG + NO_3^-.$$

Control experiments with GSH and NO_2^- alone, designed to test the possibility that air oxidation was occurring, failed to show any changes in GSH and NO_2^- concentrations except when the two were together.

Our results are in line with the well-known fact that nitroglycerin conversion to 1,2-GDN and 1,3-GDN involves the use of glutathione, but also indicate that the nitrite ions formed as a by-product of this reaction can react with glutathione to form nitrate ions,

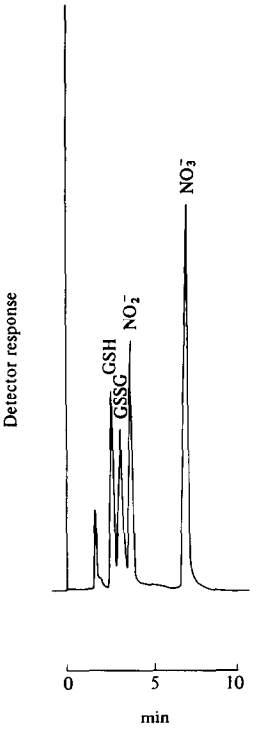

Figure 4
Chromatographic separation of reduced and oxidized glutathione, inorganic nitrite and inorganic nitrate.

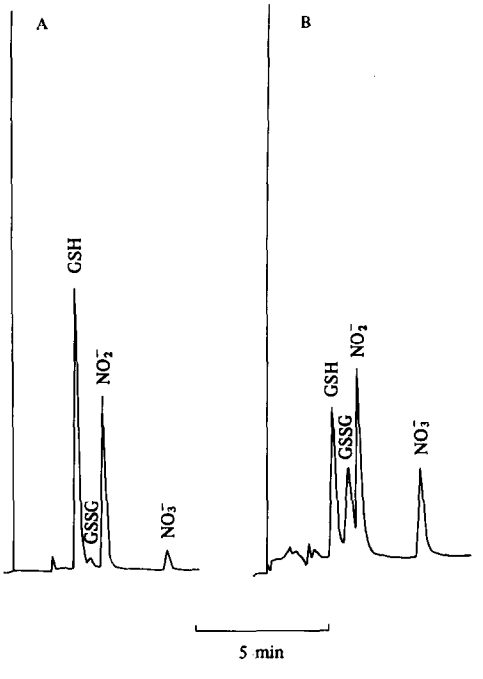

Figure 5
Chromatographic evidence for the reaction of glutathione and inorganic nitrite: (A) after 15 min reaction; (B) after 215 min reaction.

with the glutathione being converted to its oxidized form. The reaction of nitrite and glutathione occurs non-enzymatically, but may occur enzymatically at an enhanced rate. The biological significance of this reaction remains to be determined.

Acknowledgements: This work was conducted in partial fulfilment of the requirements for the M.Phil. degree of The University of London (UK) by Suher Aburawi. The investigation was carried out partly in London and partly in Gainesville, and was supported by funds provided by the Government of Libyan Peoples Jamahiriya. A preliminary report has been presented [15].

References

[1] J. R. Parratt, *J. Pharm. Pharmacol.* **31**, 801–809 (1979).
[2] P. Needleman, in *Organic Nitrates* (*Handbook of Experimental Pharmacology*, Vol. 40), pp. 57–95. Springer, Berlin (1975).
[3] M. G. Bogaert, M. T. Rosseel and A. F. deSchaepdryver, *Arch. Int. Pharmacodyn. Ther.* **76**, 458–460 (1968).
[4] J. R. Hodgson and C. Lee, *Toxicol. and Applied Pharmacol.* **34**, 449–455 (1975).
[5] P. Needleman and J. C. Krantz, *Biochem. Pharmacol.* **14**, 1225 (1965).
[6] N. H. Lee and F. M. Belpaire, *Arch. Int. Pharmacodyn. Ther.* **196**, 165–167 (1972).
[7] F. J. DiCarlo, *Drug Metabol. Rev.* **4**, 1–38 (1975).
[8] E. Boyland and B. E. Speyer, *Biochem. J.* **119**, 463–472 (1970).
[9] S. Aburawi, S. H. Curry and R. Whelpton, *Int. J. Pharmaceut.* **22**, 327–336 (1984).
[10] *British Pharmacopoeia*, p. 220, London, HMSO (1973).
[11] A. C. Bratton and E. K. Marshall, *J. Biol. Chem.* **128**, 537–550 (1939).
[12] B. Saville, *Analyst* **83**, 670–672 (1958).
[13] H. S. Buttar, A. Y. K. Chow and R. H. Downier, *Clin. Exp. Pharmac. Phys.* **4**, 1–6 (1977).
[14] W. G. Crouthamel and B. Dorsch, *J. Pharm. Sci.* **68**, 237–238 (1979).
[15] S. Aburawi and S. H. Curry, *Fed. Proc.* **43**, 560 (1984).

[*First received for review 24 September 1986; revised manuscript received 20 October 1986*]