Journal of Pharmaceutical & Biomedical Analysis
Vol. 6, No. 3, pp. 329–336, 1988
Printed in Great Britain

0731–7085/88 $3.00 + 0.00

Short Communication

Simultaneous determination of glutathione, glutathione disulphide, paracetamol and its sulphur containing metabolites using HPLC and electrochemical detection with on-line generated bromine

A. J. J. DEBETS,* R. VAN DE STRAAT,† W. H. VOOGT,* H. VOS,* N. P. E. VERMEULEN† and R. W. FREI*‡

* *Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands*
† *Department of Pharmacochemistry, Molecular Toxicology, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands*

Keywords: *HPLC; glutathione; glutathione disulphide; paracetamol; step-gradient; electrochemical detection; bromine generation.*

Introduction

Glutathione (GSH) is the major non-protein sulphydryl compound in cells of many different organisms. Large concentrations of GSH up to 10 mM are found in the liver of mammals. Apart from various roles in cellular metabolism, one of the major roles of GSH in the liver is the detoxification of electrophilic xenobiotics and metabolites [1, 2]. In the latter case GSH acts either as a reductant, with concomitant formation of oxidised GSH (GSSG), or as a nucleophile, with formation of glutathione conjugates. One example of a compound undergoing metabolic activation to a toxic metabolite is paracetamol, a widely analgesic drug which causes hepatic necrosis in animals and man upon overdosage [3]. After a normal dose of *N*-acetyl-*para*-benzoquinone imine (NAPQI), the presumed toxic metabolite formed from paracetamol is detoxified by GSH with formation of a 3-glutathionyl–paracetamol (PAR–SG) conjugate. This conjugate undergoes further metabolism and is ultimately excreted in the urine as cysteine- and *N*-acetyl–cysteine conjugates [4]. Alternatively, the NAPQI is detoxified by reduction to paracetamol with formation of GSSG [5] (Fig. 1). In view of the essential role of GSH in the detoxification of reactive or electrophilic compounds and in maintaining cellular functions, the fate of GSH in cells or organs which are exposed to toxins is of general toxicological interest.

‡To whom correspondence should be sent.

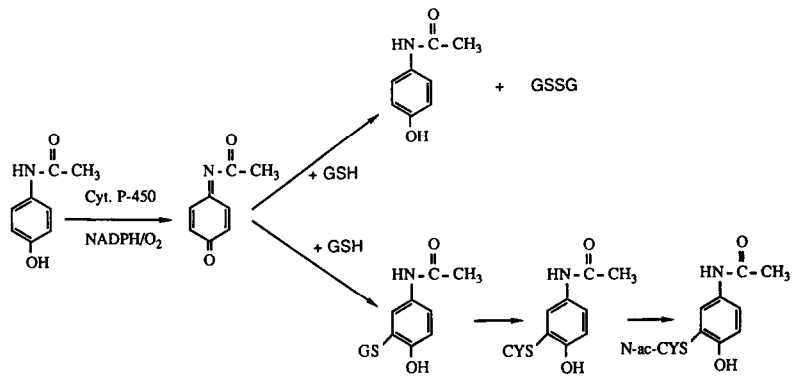

Figure 1
Schematic representation of the hepatic bioactivation and detoxication of paracetamol (PAR).

Numerous methods for determination of GSH have been reported. The original non-specific colourimetric methods were later developed into more specific enzymatic assays [6]. Recently, high performance liquid chromatography (HPLC) techniques have been described which allow relatively rapid, sensitive and selective measurements of GSH [7]. Only a few procedures for the simultaneous determination of both GSH and GSSG are described [8–12] using different derivatisation and/or detection techniques. To our knowledge no methods have been reported for the simultaneous determination of GSH, GSSG and GSH conjugates, although this could give valuable information on the fate of GSH in cells or organs.

Recently, an electrochemical detection system has been described which is capable of detection of both thiols and thio-ethers [13]. The system made use of the oxidisability of thiols and thio-ethers by elementary bromine. The general concept of this system (Fig. 2) is the on-line, post-column electrochemical production of bromine from bromide present in the mobile phase, followed by the amperometric measurement of any loss of bromine due to reactions with analytes in a reaction coil placed between electrochemical production and detection cell.

The present paper describes a sensitive and specific application of this electrochemical detection system to the simultaneous determination of GSH, GSSG and GSH conjugates in rat liver microsomes, using paracetamol as a model compound.

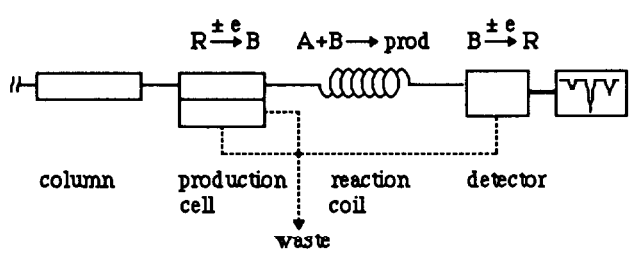

Figure 2
System with on-line electrochemical reagent production and detection. R = reagent precursor; B = reagent; A = analyte.

Experimental

Chemicals

GSH, GSSG, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, catalase, and NADP were obtained from Boehringer (Mannheim, FRG). Potassium phosphate, phosphoric acid (85%), $LiNO_3$, trichloroacetic acid, EDTA and methanol were purchased from Baker (Deventer, The Netherlands). Paracetamol was obtained from Brocacef (Delft, The Netherlands), and sodium octylsulphate from Sigma (St. Louis, USA). The 3-glutathionyl–paracetamol conjugate was synthesized as described by Potter *et al.* [14]. 3-*S*-Paracetamol–cysteine was a gift from Sterling Winthrop (UK).

Microsomes, preparation and incubation

Liver microsomes of male albino Wistar rats (180–200 g), treated 48 h before use with 3-methylcholanthrene, were prepared by ultracentrifugation as described previously [15] and stored at −180°C before use. The incubation mixtures (1 ml) consisted of 5 mM glucose-6-phosphate, 5 mM $MgCl_2$, 1 mM NADP, 0.04 IU glucose-6-phosphate dehydrogenase, 1 mM GSH, 2000 U catalase, 1 mg microsomes and 2.5 mM PAR in 100 mM potassium phosphate buffer (pH 7.4). After incubation for 10 min at 37°C, the reactions were terminated by addition of 0.5 ml 25% (w/v) cold trichloroacetic acid. The supernatant obtained after centrifugation at 3000 *g* was used for analysis.

Equipment

The chromatographic equipment consisted of an Altex 100 pump, which was set to a flow rate of 0.6 ml min^{-1} and a Rheodyne injection valve with a 20 μl loop. Separation was achieved using a 250 × 3.6 mm, 5 μm ODS Spherisorb (Phase Sep, Queensferry, UK) column. The cell used for the generation of bromine, the KOBRA cell, was constructed in the workshop of the Free University Chemistry Department and can be made available on request. Its basic concept and performance characteristics have been published [13]. A Metrohm 1096/2 cell with a 4 mm diameter Pt working electrode operating at +0.5 V vs Ag/AgCl/1 mM KCl in water was used as downstream detector. The constant current source used for bromine generation was a Tacussel GTPS bipotentiostat. A Waters absorbance detector model 440 (254 nm) was used for UV detection.

Results and Discussion

Separation

In order to simultaneously detect GSH, GSSG and the PAR–SG conjugate, chromatographic conditions described for GSH and GSSG detection were taken as starting point; ion-pairing with sodium octylsulphate was found to be necessary for sufficient retention of GSH and GSSG on the ODS column. The mobile phase described by Allison *et al.* [16] for the separation of GSH and GSSG was modified for the present system by the addition of 0.1 mM EDTA to complex free metal ions, of 0.1 M $LiNO_3$ as electrolyte, and of 1 mM KBr as reactant precursor. Using these chromatographic conditions GSH and GSSG had k′-values of 1.4 and 5.8, respectively. Paracetamol, the model drug used in this study, could also be detected by the bromine generating system, presumably because of its relatively low oxidation potential ($E_{1/2}$ = 0.54 V). Paracetamol had a k′-value of 7.1. However, under these chromatographic conditions the PAR–SG

conjugate and PAR–Cyst conjugate had extremely high k′-values, namely 36.5 and 20.0 respectively. This problem could be solved using a single step gradient after the elution of GSH, GSSG and PAR. By instantaneously increasing the methanol content from 4 to 10%, the k′-values of PAR–SG and PAR–Cyst were reduced to 21.2 and 16.5, respectively. A typical chromatogram of a sample of GSH, GSSG, paracetamol, PAR–Cyst and PAR–SG using the bromine generating system and the single step gradient is shown in Fig. 3A. A simultaneously measured UV-trace (Fig. 3B) in which only paracetamol and its conjugates were detected, clearly illustrates the advantage of the bromine generating and amperometric detection system.

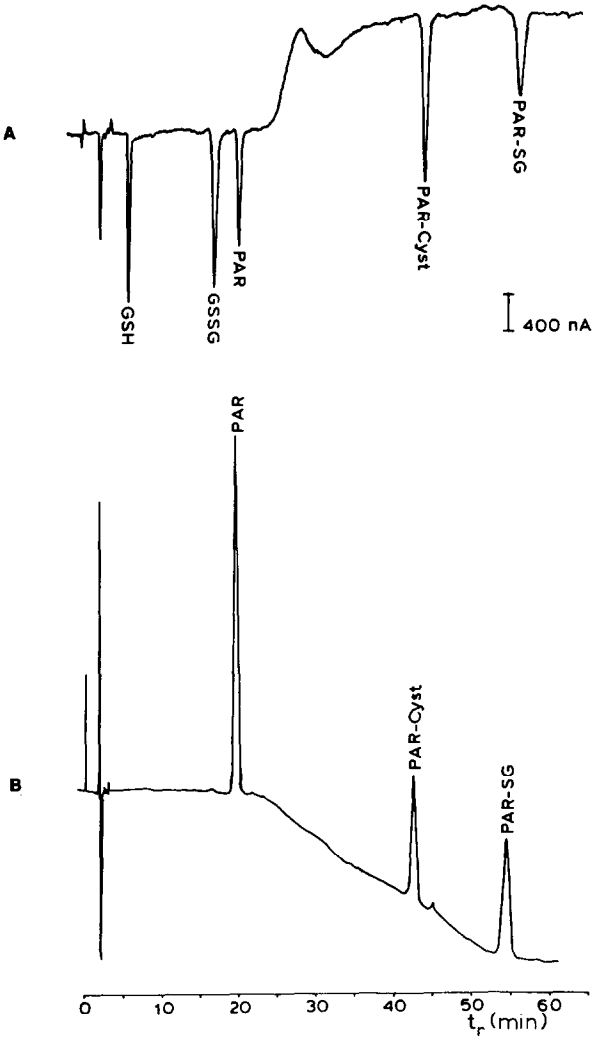

Figure 3
Chromatogram of a standard solution of PAR–SG (2.5×10^{-5}M), PAR–Cyst (10^{-5}M), PAR (3×10^{-5}M), GSSG (10^{-5}M) and GSH (10^{-5}M). Detection systems: (A) Br_2-generation; (B) UV/Vis, 0.05 a.u.f.s. The ODS column was eluted isocratically with mobile phase, containing 5×10^{-2}M phosphate buffer (pH = 3), 10^{-1}M $LiNO_3$, 10^{-3}M KBr, 10^{-4}M EDTA and 4% methanol, for 20 min, followed by a single step gradient increasing the methanol content from 4 to 10%.

Detection

The sensitivity of detection of the bromine generating system is dependent on the reaction kinetics of the analyte with bromine. The optimum reaction conditions were determined by means of repeated on-column injections of standards of GSH, GSSG, mobile phase as described above, or paracetamol–3-cysteine conjugate (PAR–Cyst), as a representative paracetamol–thioether conjugate. For the latter the mobile phase contained 5×10^{-2}M phosphate buffer (pH = 2), 10^{-1}M $LiNO_3$, 10^{-3}M KBr, 10^{-4}M EDTA and 30% methanol. Peak areas were measured with varying generation currents, reaction coil lengths and reaction temperatures.

The problem of a simultaneous determination of GSH, GSSG and thioether conjugates of PAR is not only to find optimum common conditions for chromatography and detection of all components, but also the fact that their concentrations in biological samples differ considerably. In view of these facts, a bromine generating current of 100 μA was used throughout the experiments. This has the advantage of a wider linear range of detection due to a higher coulometric yield. In principle, however, a disadvantage is a lower signal to noise ratio [13]. A decreased generation current showed no dramatic increase in signal to noise ratio for GSH and GSSG (Fig. 4). For PAR–Cyst, however, a decreased generation current increased the signal to noise ratio significantly which made low detection limits possible.

The dependence of the sensitivity of detection on the reaction time of the analytes with bromide was studied by varying the length of the reaction coil. Variation of the reaction time from 1.5 s to 10 s had no effect on GSH and GSSG detection. However, in the case of PAR–Cyst the peak area doubled by using an about 10-fold longer reaction coil. Since the increase was most pronounced at 4.2 s (reaction coil of 70 μl) and since the band broadening under these circumstances was still negligible, further experiments were performed with a reaction coil of 70 μl.

Upon elevation of the reaction temperature the peak areas of GSH and GSSG, but not PAR–Cyst, increased to a maximum of 230% on a traject from 25°C to 60°C. The rate of this increase was greater for GSSG than for GSH.

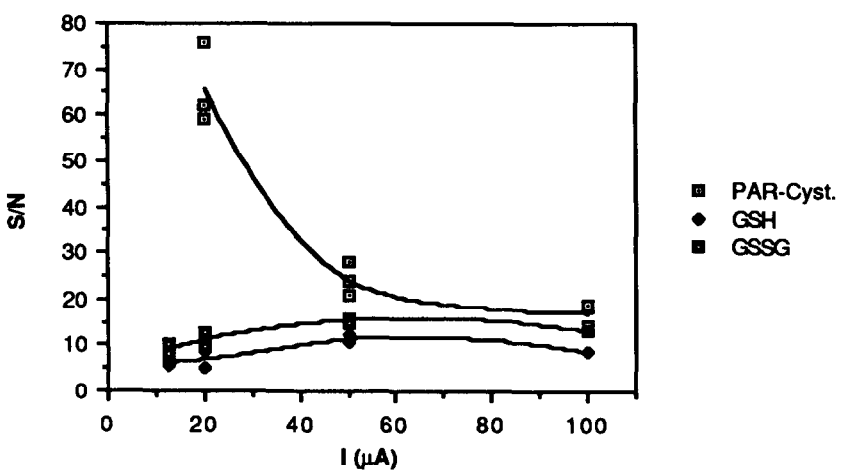

Figure 4

Dependence of the signal to noise ratio (S/N) on the generating current (Ig). The mobile phase for the GSH (1.5×10^{-6}M) and GSSG (1.5×10^{-6}M) data contains 5×10^{-2}M phosphate buffer (pH = 3), 10^{-1}M $LiNO_3$, 10^{-3}M KBr, 10^{-4}M EDTA and 4% methanol; for the PAR–Cyst data 5×10^{-2}M phosphate buffer (pH = 2), 10^{-1}M $LiNO_3$, 10^{-3}M KBr, 10^{-4}M EDTA and 30% methanol.

Using the conditions described above, linearity, reproducibility and detection limits were determined. The detection of both GSH and GSSG was linear in the range of 10^{-6}–10^{-5}M, showing a steeper slope of the GSSG (slope = 1184 mAs/μM; $r = 0.998$) curve in comparison with the GSH (slope = 624 mAs/μM; $r = 0.997$) curve, since the two sulphur atoms of GSSG cause a greater loss of bromine upon reaction when compared to GSH [17]. Detection limits of 10 pmol for GSH (150 ng ml^{-1}) and 4 pmol for GSSG (120 ng ml^{-1}) were determined under these circumstances (S/N = 3). Although conditions used were not optimal, these detection limits are already in the same range as described for other methods for the simultaneous determination of GSH and GSSG (Table 1). Using optimum detection conditions specific for determination of GSH and GSSG even lower detection limits could be achieved [12]. Standard solutions (5×10^{-6}M) for both GSH and GSSG can be determined with a reproducibility better than 1.5% ($n = 8$). Even in samples in which the GSH to GSSG ratio was 99:1, which typically can occur in biological samples and which causes problems with for instance the Hg/Au method [18], the determination of GSSG was still highly reproducible. The detection limit of PAR–Cyst with the bromine system was 1 pmol (13.5 ng ml^{-1}, S/N = 3) with a generation current of I = 20 μA. In practice the UV detection of this compound appeared to be 1 order of magnitude lower.

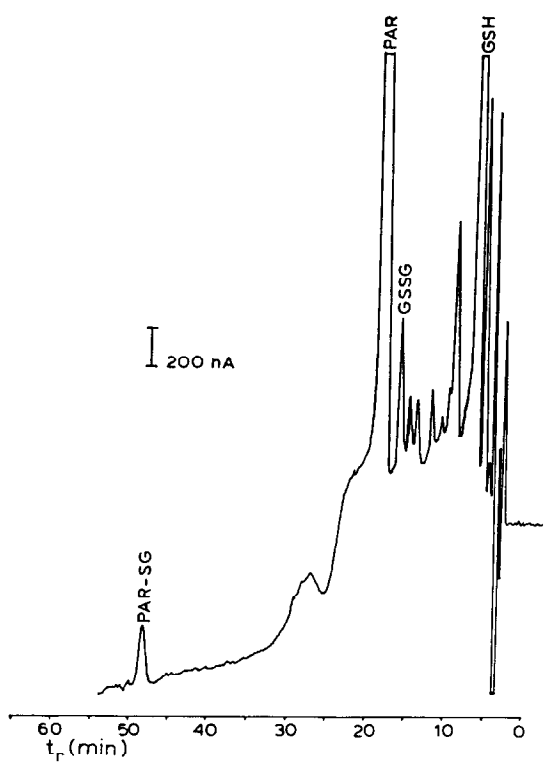

Figure 5
Chromatogram of a rat liver microsomal sample. Incubations were performed as described in the experimental section. Separation procedure, electrochemical detection and mobile phase were as given in Fig. 3.

Application to a biological sample

Rat liver microsomal metabolism of paracetamol in the presence of GSH was selected as a typical biological sample to study the applicability of the proposed chromatographic system. As shown in Fig. 5, after aerobic incubation of paracetamol, NADPH, GSH and liver microsomes, both GSSG and PAR–SG conjugate were detected. These products were not present at all (PAR–SG) or present in considerably lower amounts (GSSG) in control incubations.

Conclusions

A method for the simultaneous detection of GSH, GSSG and GSH conjugates (or metabolites thereof) using an on-line bromine generating and an amperometric detection system is presented. The detection of GSH and GSSG by this approach has the advantage over other procedures in that no sample derivatisation and no expensive and complex post-column reactors are needed and its response is reproducible even in the case of wide variations in the concentrations of analytes. In addition, due to its inherent selectivity the sample preparation procedure can be rather simple when analysing biological fluids. The sensitivity is at least comparable to that of other detection methods (Table 1). Furthermore, GSH conjugates can also be detected even if gradient steps are required. Therefore this method may be more generally applicable in the study of the fate of GSH and GSH conjugates in biological systems.

Table 1
Comparison of HPLC detection techniques for GSH and GSSG in biological systems

Detection method	Detection limits GSH (pmol)	GSSG (pmol)	Ref.
Post-column derivatisation using *o*-phthalaldehyde with 2-mercaptoethanol	10	10	[8]
Enzymatic recycling procedure	2	2	[9]
Dual Au/Hg-electrodes system	3.5	5.7	[10]
Pre-column derivatisation with iodoacetic acid and 1-fluoro-2,4-dinitrobenzene	<100*	<100*	[11]
Br_2-generation	10	4	

*Lowest reported concentration in the calibration curve.

References

[1] *Functions of Glutathione: Biochemical Physiological, Toxicological and Clinical Aspects* (A. Larson, A. Holmgren, S. Orrenius and B. Mannervik, Ed.). Raven Press, New York (1983).
[2] N. Kaplowitz, T. Y. Aw and M. Ookhtens, *Ann. Rev. Pharmacol. Toxicol.* **25**, 715–744 (1985).
[3] L. F. Prescot, *Drugs* **25**, 290–314 (1983).
[4] J. A. Hinson, in *Reviews in Biochemical Toxicology* (E. Hodgson, J. R. Bend and R. M. Philpot, Eds), Vol. 2, pp. 103–129. Elsevier, Amsterdam (1980).
[5] E. Albano, M. Rundgren, P.J. Harvison, S. D. Nelson and P. Moldeus, *Molec. Pharm.* **28**, 306–311 (1985).
[6] P. C. Jocelyn, *The Biochemistry of the functional of the SH group*. Academic Press, New York (1972).
[7] D. Perret and S. Rudge, *J. Pharmac. Biomed. Anal.* **3**, 3–27 (1985).

[8] D. A. Keller and D. B. Menzel, *Anal. Biochem.* **151**, 418–423 (1983).
[9] A. J. Alpert and H. F. Gilbert, *Anal. Chem.* **144**, 553–562 (1985).
[10] L. A. Allison and R. E. Shoup, *Anal. Chem.* **55**, 8–12 (1983).
[11] D. J. Reed, J. R. Babson, P. W. Beatty, A. E. Brodie, W. W. Ellis and D. W. Potter, *Anal. Biochem.* **106**, 55–62 (1980).
[12] K. Isaksson, J. Lindquist and K. Lundström, *J. Chromatogr.* **324**, 333–342 (1985).
[13] W. Th. Kok, U. A. Th. Brinkman and R. W. Frei, *Anal. Chim. Acta* **162**, 19–32 (1984).
[14] D. W. Potter, P. W. Miller and J. A. Hinson, *Molec. Pharmac.* **39**, 155–162 (1985).
[15] R. van de Straat, J. de Vries, T. Kulkens, A. J. J. Debets and N. P. E. Vermeulen, *Biochem. Pharmac.* **35**, 3693–3699 (1986).
[16] L. A. Allison, J. Keddington and R. E. Shoup, *J. Liq. Chromatogr.* **6**, 1785–1798 (1983).
[17] F. Rauwel and D. Thevenot, *Bioelectrochem. Bioenerg.* **3**, 284–301 (1976).
[18] S. M. Lunte and P. T. Kissinger, *J. Liq. Chromatogr.* **8**, 691–706 (1985).

[*Received for review 3 August 1987; revised manuscript received 23 September 1987*]