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Measurement of reduced and oxidized glutathione in cultures of adult rat hepatocytes

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ABSTRACT

A reversed-phase ion-exchange high-performance liquid chroamtographic technique, suitable for the separate measurement of reduced (GSH) and oxidized (GSSG) glutathione in cultures of adult rat hepatocytes, is described. A commercially available Nucleosil 120-7NH_2 column was used. A complete run took *ca.* 22 min. The retention times for GSH and GSSG were 10.6 and 12.7 min, respectively, providing a resolution coefficient of 1.4. The coefficients of variation for GSH and GSSG were *ca.* 5 and 25%, respectively, for freshly isolated hepatocytes, and 16 and 15%, respectively, for 24-h cultured hepatocytes. The detector response was linear as a function of GSH and GSSG concentration and the hepatocytes concentration studied. Addition of up to 1.5 mg/ml bovine serum albumin to the culture medium had no effect on the linearity. The recovery for standards, ranging from 0 to 150 nmol of GSH or GSSG per millilitre in the presence of hepatocytes, was 98% for GSH and 80% for GSSG. The detection limit of the method was between 0.5 and 1.0 nmol of GSH and GSSG per millilitre. In cultured rat hepatocytes, the GSH content increased during the first 24 h of culture, followed by a slow decrease. After six days of culture, the GSH content was less than 50% of the value found for freshly isolated hepatocytes. GSSG was present in cultured rat hepatocytes in only small amounts and becomes unmeasurable after four days of culture.

INTRODUCTION

In previous studies [1–3], it has been shown that the activity of glutathione S-transferase and its isoenzyme pattern in cultures of adult rat hepatocytes can be affected by many soluble factors. In order to analyse these effects in more detail, it is of interest to study the glutathione content that is available in the medium and inside the hepatocytes. It is not yet clear if these values undergo changes as a function of culture time and/or of culture conditions. Therefore, a suitable method capable of measuring both the reduced (GSH) and oxidized (GSSG) forms of glutathione is necessary. Several methods dealing with the measurement of both forms together have been described [4,5]. In some cases, one component could be determined together with the total amount of both forms, thus allowing the calculation of the remaining component [6]. However, few methods offer the possibility of measuring the reduced and oxidized forms separately, during the same procedure. The best known method is the high-performance liquid chromatographic (HPLC) technique first described by Reed *et al.* [7] and modified by

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Fariss and Reed [8]. In order to make this method more suitable for practical use, the following modifications have been introduced: (1) the use of a commercially available column; (2) the adaptation of the HPLC gradient to the column used; (3) the development of a procedure suitable for the measurement of GSH and GSSG in cell cultures.

The linearity of the method as a function of time and protein content, and the reproducibility, recovery and detection limits have been determined. The time course of GSH and GSSG contents in cultures of adult rat hepatocytes has been measured using this modified method.

EXPERIMENTAL

Animals

Male Sprague–Dawley rats, body weight 150–200 g, were obtained from Iffa Credo, (Brussels, Belgium). Male Wistar rats, body weight 150–200 g, were provided by the Animalarium, Vrije Universiteit Brussel (Brussels, Belgium). All animals had free access to water and food.

Chemicals and reagents

Leibovitz, Medium 199, minimum essential medium and fetal calf serum (FCS) were obtained from Gibco (Paisley, U.K.). Bovine serum albumin (BSA), sodium acetate trihydrate, 2,4-dinitrofluorobenzene (FDNB), iodoacetic acid (IAA), 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), insulin, kanamycin monosulphate (781 μg/mg), penicillin (1662 U/mg), streptomycin sulphate (750 U/mg) and collagenase (Type 1, 300 U/mg) were purchased from Sigma (St. Louis, MO, U.S.A.). Trypan blue was provided by Flow Labs. (Zellik, Belgium), hydrocortisone hemisuccinate by Roussel (Brussels, Belgium), pentobarbital (Nembutal®) by Sanofi, (Ceva, Brussels, Belgium), "Bio Rad protein assay kit®" by Bio Rad Labs. (Munich, Germany), and ethylenediaminetetraacetic acid, disodium salt dihydrate (EDTA) by Aldrich Chemie, (Steinheim, Germany).

Materials

Sterile Petri dishes (28 cm^2) were obtained from Falcon, Becton Dickinson Labware (Oxnard, CA, U.S.A.) and the Nucleosil 120-7 NH_2 column (100 mm × 4 mm I.D.), used for HPLC, was purchased from Macherey-Nagel, (Düren, Germany). The 5000 liquid chromatograph was obtained from Varian, (Sunnyvale, CA, U.S.A.), the LC–UV detector from Pye Unicam (Cambridge, U.K.), and the integrator from Intersmat (Brussels, Belgium).

All other compounds, reagents and materials used were readily available commercial products.

Isolation and culture conditions of rat hepatocytes

Hepatocytes were isolated as previously described [9]. Cell integrity was tested by Trypan blue exclusion, and was 82–92%.

Hepatocytes were seeded at a density of $1.5 \cdot 10^6$ cells per 28-cm^2 Petri dish in 4 ml of standard medium with 10% FCS. The standard medium consisted of a mixture of 75% minimum essential medium and 25% medium 199 containing 1 mg/ml BSA, 10 μg/ml bovine insulin, 50 μg/ml kanamycin monosulphate, 50 μg/ml streptomycin sulphate and 4.5 μg/ml penicillin. The medium was first changed 4 h after cell seeding, and every day thereafter. The renewing media were supplemented with $7 \cdot 10^{-5}$ *M* hydrocortisone hemisuccinate.

Protein assay

Proteins were measured using a "Bio Rad protein assay kit" with BSA as a standard.

Derivatization procedure

The derivatization procedure and the HPLC analysis were based on the method described by Reed *et al.* [7] and modified by Farris and Reed [8].

The incubation medium was removed and stored. The cells were washed twice with phosphate-buffered saline (PBS), and scraped off with 2 ml of PBS and stored. The samples thus obtained were kept on ice and homogenized by ultrasonification for 1 min.

A 1-ml volume of each sample was deproteinized by adding dropwise 0.5 ml of 10% perchloric acid with 2 m*M* EDTA under constant stirring, and the remaining sample was used for protein determination. Proteins were removed by centrifugation, and to a 0.5-ml volume of the supernatant 0.05 ml of 20 mg/ml IAA solution was added followed by 0.45 ml of a KOH (2 *M*)–$KHCO_3$ (2.4 *M*) solution. After incubation for 15 min at room temperature in the dark, 1 ml of 1% FDNB in ethanol was added. This reaction mixture was kept in the dark at room temperature for 24 h before analysis or storage (in the dark at 0–4°C). Standard solutions of GSH and GSSG were subjected to the same derivatization procedure. Aliquots of 50 μl were analysed by HPLC.

Detection

The 2,4-dinitrophenyl derivatives were detected spectrophotometrically at 365 nm.

HPLC solvent and gradient system

A commercially available Nucleosil 120-7 NH_2 column, suitable for the separation of saccharides, phenols and isomers, aminophenols, amines and vitamins, was used. Solvent A consisted of methanol–water (4:1, v/v). Solvent B was obtained by mixing 272 g of sodium acetate trihydrate, 122 ml of water and 378 ml of glacial acetic acid, then adding 200 ml of the resulting solution to 800 ml of solvent A.

The gradient for HPLC analysis started after a 5-min isocratic period at 25% B and increased linearly to 95% over 8 min. After another 4-min isocratic period, the proportion of solvent B was reduced to 25% in 0.1 min. The flow-rate was 2.0 ml/min.

RESULTS

HPLC technique

Fig. 1A and B show the separation between GSH and GSSG for a standard mixture and a biological sample, respectively. A complete run takes *ca.* 22 min. The separation is satisfactory, as shown by the resolution coefficient of 1.4. The retention times, 10.6 and 12.7 min for GSH and GSSG, respectively, when a new

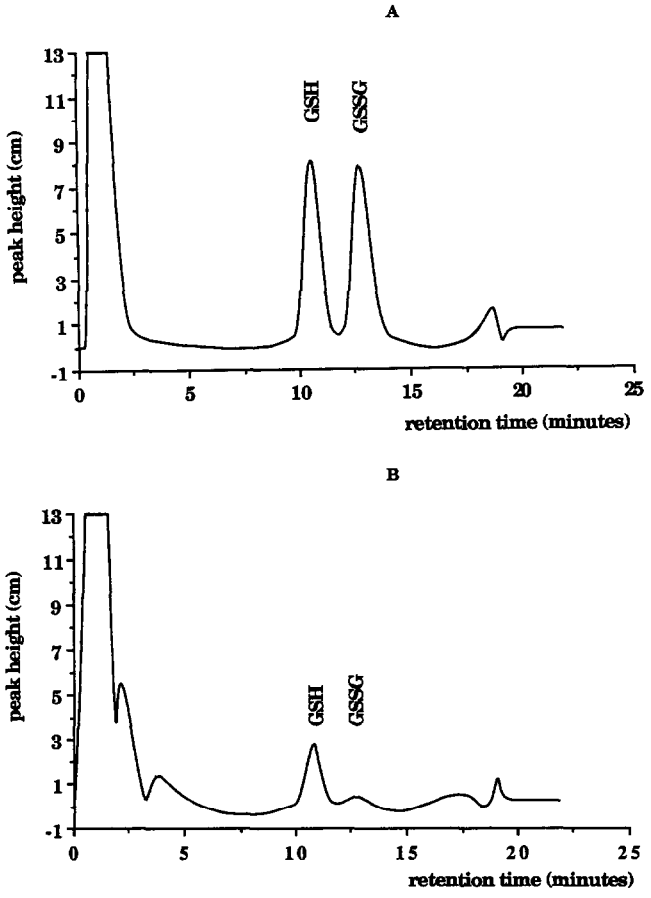

Fig. 1. Typical chromatograms of the separation between GSH and GSSG. A standard mixture of 60 nmol/ml GSH and GSSG (A) and a sample (hepatocytes) taken after two days of culture (B) were subjected to derivatization and HPLC analysis, respectively.

column is used, decrease as the column ages although the resolution between the two peaks is not really affected (1.3 after *ca.* 250 analyses). It is only after *ca.* 300 analyses that the separation between the solvent peak and the GSH peak is lost.

The reproducibility of the complete analysis was checked with freshly isolated hepatocytes and mean GSH and GSSG contents of 14.0 ± 0.7 nmol per 10^6 cells [coefficient of variation (C.V.) = 5%; n = 9] and of 0.7 ± 0.6 nmol/10^6 cells (C.V. = 25%; n = 9), respectively were found. For 24-h cultured hepatocytes, C.V. of 16% (n = 8) and 15% (n = 4) were measured, respectively.

Fig. 2A shows the linearity of he area under the curve (AUC) of each peak as a function of its concentration for standard mixtures of GSH and GSSG in water. Correlation coefficients of 1.00 and 0.98 were found for GSH and GSSG, respectively (n = 3). When freshly isolated hepatocytes were used, it was clear that

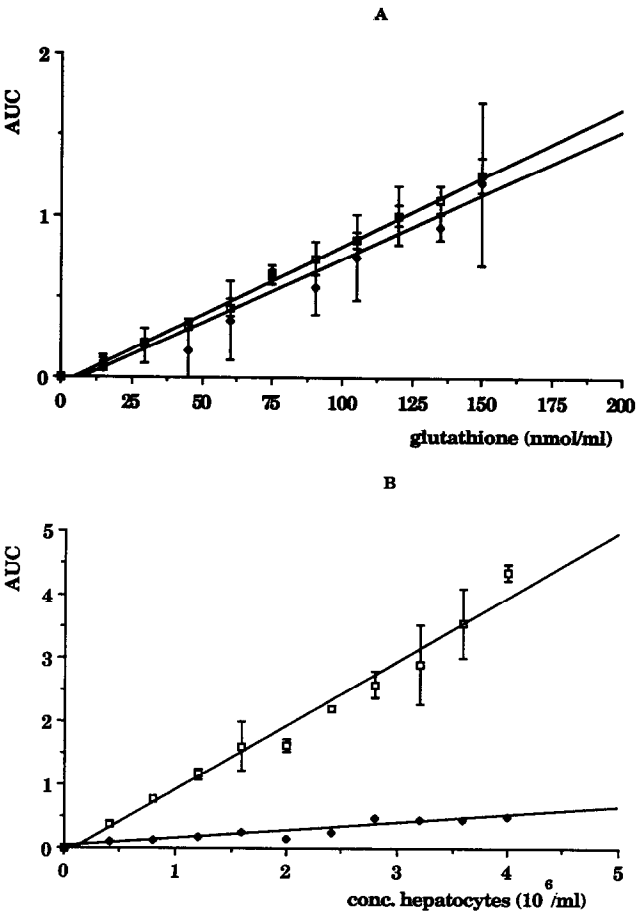

Fig. 2. (A) Linearity of the HPLC response as a function of the GSH and GSSG concentrations (n = 3); (□) $y = -0.0373 + 0.0085x$, r = 1.00 for GSH; (◆) $y = -0.0613 + 0.0079x$, r = 0.98 for GSSG. (B) Linearity of the HPLC response as a function of the hepatocyte concentration: (□) $y = -0.0949 + 1.0083x$, r = 0.99 for GSH (n = 3); (◆) $y = 0.0215 + 0.1226x$, r = 0.93 for GSSG (n = 1).

satisfactory correlation coefficients were obtained for GSH and GSSG (0.99 and 0.93, respectively, with $n = 3$) (Fig. 2B).

The addition of BSA (1.5 mg/ml) to GSH or GSSG standards had no effect on the linearity, as shown in Fig. 3A and B.

To check the recovery, standards ranging from 0 to 150 nmol GSH or GSSG per millilitre, were analysed with and without the addition of $1.5 \cdot 10^6$ hepatocytes per millilitre. In the presence of hepatocytes, mean recoveries of 98% (C.V. = 11%) and 80% (C.V. = 17%) were found for GSH and GSSG, respectively ($n = 3$).

When 0–2 mg/ml BSA was added to these standard mixtures, it appeared that all the recoveries were within the standard deviation range of the mixtures without BSA. Indeed, the mean recoveries were 92 ± 15 and 100 ± 22% for GSH

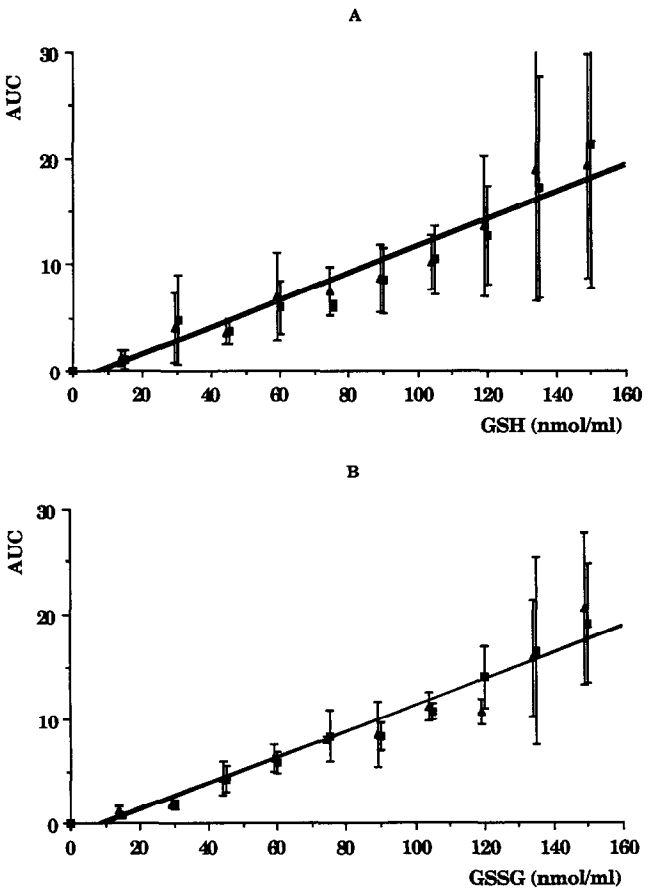

Fig. 3. Linearity of the GSH (A) or GSSG (B) determinations in the presence (▲) or absence (■) of 1.5 mg/ml BSA ($n = 2$). GSH: (■) $y = -1.12578 + 0.128x$, $r = 0.96$; (▲) $y = -1.0183 + 0.1276x$, $r = 0.97$. GSSG: (■) $y = -1.4075 + 0.1277x$, $r = 0.99$; (▲) $y = -1.1906 + 0.1237x$, $r = 0.97$.

and GSSG, respectively (n = 9). The detection limit of the method used ranged between 0.5 and 1.0 nmol GSH or GSSG per millilitre, depending on the background noise.

GSH and GSSG contents in cultured hepatocytes

Fig. 4 shows the GSH and GSSG contents of cultured rat hepatocytes, determined by the method described here, as a function of culture time. It appears that during the first 24 h of culture the GSH content increases and then slowly decreases. After six days the GSH level represents less than 50% of the value measured for freshly isolated hepatocytes. GSSG is present in cultured rat hepatocytes in only small amounts and becomes unmeasurable after four days of culture.

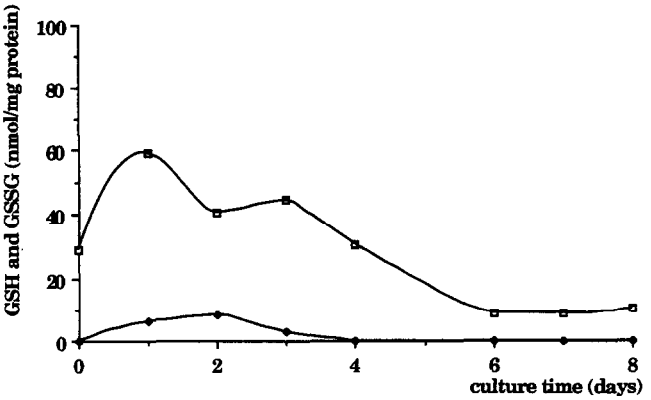

Fig. 4. GSH (□) and GSSG (◆) contents of adult rat hepatocytes in conventional culture in function of culture time. One typical experiment out of three is shown.

DISCUSSION

The method proposed here is a modification of the technique described by Reed *et al.* [7]. A commercially available column, Nucleosil 120-7 NH_2, is used for the HPLC separation of GSH and GSSG in biological samples, thus avoiding time-consuming preparation of 3-aminopropylsilane-derivatized silica columns [7]. The retention times are considerably shorter than those reported in the original method [7], allowing more analyses per day.

A derivatized standard mixture of both components needs to be injected every day in order to establish the AUCs and the retention times, because the latter tend to decrease as the column ages. About 300 analyses with clean separation and no peak tailing could be carried out with one column.

The reproducibility, linearity, recovery and detection limits of the method

proposed are comparable with those of the previous method [7], and the addition of BSA had no effect on the linearity or the recovery.

The applicability of the method was shown by analysing the GSH and GSSG content of cultured rat hepatocytes. The values obtained for freshly isolated hepatocytes are within the range of data published previously [7,8,10]. Several investigators [8,26–29] have reported similar results, although others have published higher values [7,10–25]. Many factors, such as oxygenation during perfusion and the presence of substances that cause cellular damage [11,30], may influence the GSH and GSSG levels measured and may explain the initial increase of GSH, observed in all the samples analysed. An initial increase was also observed by Meredith [31], who cultured hepatocytes in hormonally defined media. The GSSG content of rat hepatocytes in culture is much lower than the GSH content, which is to be expected under normal conditions [32]. It is only during oxidative stress that high intracellular GSSG levels are reached and render the cells more oxidized [32].

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