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Determination of intracellular glutathione in human skeletal muscle by reversed-phase high-performance liquid chromatography

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Abstract

A chromatographic method for the specific determination of cellular low molecular mass thiols has been applied to human muscle tissue. The method is based on the derivatisation of thiols using monobromobimane, which is a specific reagent for the sulphydryl group. The glutathione and cysteine bimane adducts were separated by reversed-phase HPLC, whilst quantitation of the cysteine and glutathione adducts was achieved by fluorescence spectroscopy. The method was found to yield a quantitative recovery of glutathione (ca. 96%), to be sensitive (down to 20 pmol glutathione/per injection) and reveal a low intra-individual coefficient of variation (C.V. $< 5\%$) of the glutathione concentrations in human skeletal muscle. The concentrations of reduced and total glutathione were 1320 ± 37 μmol/kg wet weight (mean $\pm$ S.E.M.) and 1525 ± 66 μmol/kg wet weight, respectively. The method was also applied to tissues from nine healthy volunteers to determine if fluctuations in glutathione level occurred over a 24-h period. No diurnal variation of glutathione level in human skeletal muscle was observed.

1. Introduction[1]

The tripeptide glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) is the predominant intracellular non-protein thiol. It plays an important role in the body as an intracellular reductant and is also involved in the synthesis of proteins and DNA, in amino acid transport [1,2] and in the scavenging of reactive oxygen species [3].

Experimental studies in rats and clinical studies in humans, concerning the metabolism and turnover of GSH in liver, kidney, erythrocytes and other tissues [1,4–10], have all indicated that a variety of exogenous and endogenous factors can influence the intracellular levels of GSH in these different tissues. However, little

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[1] Abbreviations used: GSH, reduced glutathione; GSSG, oxidised glutathione; CySH, cysteine; γ-GT, γ-glutamyl transpeptidase; mBBr, monobromobimane; DTT, dithiothreitol; SSA, sulphosalicylic acid; HPLC, high-performance liquid chromatography.

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is known concerning GSH metabolism and redox status in human skeletal muscle. The presence of GSH in the skeletal muscle of animals has long been recognised and an association has been shown between intracellular proteolysis and GSH redox status [11]. Prolonged GSH depletion in skeletal muscle leads to muscle mitochondrial damage and muscle degeneration [9]. Thus, the ability to monitor GSH metabolism in human skeletal muscle has potentially important clinical applications in studying the changes which occur in the tissue as a result of critical illness, or ischemia and reperfusion in surgery.

A number of methods are available for the determination of reduced glutathione in cells. These are often based on reactions with the sulphydryl group, e.g. Ellman's reagent or more recently, monobromobimane (mBBr) [12]. In the present study we report the application of an established HPLC method [5], based on the use of the sulphydryl-reactive reagent mBBr for the determination of thiol redox status, to the determination of human skeletal muscle glutathione in healthy volunteers. The method was also used to investigate possible diurnal variation in human muscle tissue during a 24-h period. Simultaneously, we have used material from the same muscle biopsy to determine the concentrations of the constituent amino acids of GSH.

2. Experimental

2.1. Chemicals

Monobromobimane ($C_{10}H_{11}BrN_2O_2$) was obtained in >99% purity from Calbiochem (La Jolla, CA, USA). Glutathione and cysteine of >99% purity, *N*-ethylmorpholine of 99% purity, dithiothreitol and amino acid standard solution were obtained from Sigma (St. Louis, MO, USA). Acetonitrile (HPLC grade), sulphosalicylic acid and sodium bicarbonate ($NaHCO_3$) were obtained from Merck (Darmstadt, Germany). Lithium citrate buffers for amino acids analysis were purchased from Pharmacia LKB Biochrom (Cambridge, U.K.).

2.2. Subjects

Nine healthy male volunteers participated in the study (age 24 ± 4.3 years, height 186.6 ± 8.0 cm, weight 76.4 ± 8.0 kg). Normal physical activity and food intake conformed with their usual habits during the 24-h study period.

The nature, purpose and potential risks of the experimental procedures were explained to the subjects before obtaining their voluntary consent. The research protocol was approved by the Ethics Committee of the Karolinska Institute, Stockholm, Sweden.

2.3. Tissue sampling and preparation

Percutaneous muscle biopsies were taken using Bergström's biopsy needle [13], from the lateral portion of the quadriceps femoris muscle, 15–20 cm above the knee, after applying local anaesthesia to the skin. Four biopsies were taken from each individual at four time points during the day (at 08.00 a.m., 14.00 p.m., 20.00 p.m., and 08.00 a.m.). The biopsy specimen was divided into small pieces. Each piece was weighed three times within 25 s on an automated electrobalance (Cahn 29, Cahn Instruments, Cerritos, CA, USA) and the wet weight was extrapolated to zero time. Muscle pieces weighing 40–60 mg were used for GSH determination and pieces of 20–40 mg for the determination of amino acids. After weighing, the muscle pieces were immediately frozen (within 2 min of sampling) in liquid nitrogen and stored at −80°C prior to analysis.

For GSH determination the muscle tissues were homogenised within 30 min of sampling in 6.5% (w/v) sulphosalicylic acid (SSA) solution in a glass homogenizer on ice and then centrifuged at 3000 *g* for 15 min at 4°C. The pH of the supernatant was adjusted to neutrality with excess $NaHCO_3$ powder and the sample was derivatised directly. For the determination of amino acids, the muscle sample was homogenised in 6.5% (w/v) SSA containing nor-leucine as internal standard on ice and then centrifuged at 3000 *g* for 15 min at 4°C.

The pH of the supernatant was adjusted to 2.2 using 3 *M* LiOH. The supernatant was frozen prior to analysis.

2.4. Derivatisation procedure

The derivatisation procedure was performed as described previously by Cotgreave and Moldéus [5]. The fluorescent labeling of thiols with monobromobimane is indicated as the chemical equation: $C_{10}H_{11}BrN_2O_2 + GSH \rightarrow C_{10}H_{11}SGN_2O_2 + HBr$. Briefly, a 100-$\mu$l sample of GSH/CySH standards or SSA-soluble fraction from muscle biopsies were mixed with mBBr (8 m*M* in sodium *N*-ethylmorpholine pH 8.0, 100 μl), and allowed to react for 5 min in the dark before the reaction was stopped by the addition of 10 μl 100% SSA. Aliquots of the derivatised muscle samples were filtered using a 0.22-μm filter and applied to the HPLC column for the determination of thiol bimane adducts.

Total GSH (GSH + GSSG) and cysteine (CySH + cystine) were also evaluated by the present method by performing a reduction step of GSSG and cystine with dithiothreitol (DTT) after protein precipitation. Briefly, a 100-μl portion of neutralized muscle supernatant was treated with 10 μl 50 m*M* DTT, mixed and allowed to stand at room temperature for 30 min and then derivatised with 100 μl of 20 m*M* mBBr in the dark for 5 min. The reaction was stopped by acidifying with 10 μl of 100% SSA.

A control validation of thiol recovery was performed by spiking known amounts of GSH and CySH (final concentrations of 50 μM and 100 μM) into the 6.5% SSA solution in which the muscle biopsy was homogenised. A second piece of muscle from the same biopsy was homogenised in the buffer solution without addition of GSH or CySH in order to determine the endogenous concentrations of the respective thiols. The homogenates were then further treated as described above. The actual concentration of thiol spiked into the sample was determined by derivatising the SSA solution in the same way as for the standard samples.

2.5. Chromatographic conditions

The HPLC separation of low molecular mass thiol-bimane adducts was achieved on a Supelcosil LC-18 octadecylsilyl silica column (150 × 4.6 mm I.D., 3 μm particle size) reversed-phase resin, followed by fluorimetric detection at an excitation wavelength of 394 nm and an emission wavelength of 480 nm. The system consisted of a Waters 625 LC pump system, a Waters 470 scanning fluorescence detector and a Waters 715 Ultra WISP sample processor (Millipore, Milford, MA, USA). Elution solvent A was 9.0% (v/v) aqueous acetonitrile containing 0.25% (v/v) acetic acid and 0.25% (v/v) perchloric acid of apparent pH 3.71 and elution solvent B was 75% (v/v) acetonitrile. The elution program consisted of 100% solvent A for 7 min, followed by 100% solvent B for 4 min to elute matrix interferences, and returning to solvent A for re-equilibration for 7 min at a flow-rate of 1.0 ml/min.

The free amino acid concentration of skeletal muscle biopsies, with the exception of cysteine, was measured by ion-exchange chromatography [14], using an Ultropac 8 Lithium form ion-exchange column (202 × 4.6 mm I.D., 8 μm particle size) in an automated amino acid analysis system (Alpha Plus, LKB Pharmacia, Stockholm, Sweden) using lithium citrate buffers. *o*-Phthaldialdehyde (OPA) was used for post-column derivatisation and fluorimetric detection (Shimadzu RF-535) at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. Cysteine was determined by derivatisation with monobromobimane as described above rather than OPA, since we considered this method to be more selective.

2.6. Data expression and analysis method

All data are presented as mean values ± S.E.M. The statistical analyses were performed with one-factor analysis of variance (ANOVA) for repeated measures. The different study time points were treated as repeated measures.

A *p*-value (two-tailed) of less than 0.05 was considered significant.

3. Results

3.1. Sensitivity and reproducibility of the assay

As previously described [5], the limits of detection of both CySH and GSH as their bimane adducts by this method were 10 pmol per injection. However, for calibration purposes, the minimum concentration of each thiol which may be reliably assayed by these procedures was set at 20 pmol per injection. Standards were routinely diluted from a stock standard solution of 10 mM CySH and GSH diluted in SSA solution. Values obtained from ten independent calibrations over the range of 20 pmol–4 nmol per injection yielded a coefficient of variation for the slope constant of less than 4% for both CySH and GSH. Recoveries were calculated for three different concentrations of CySH and GSH (1.0, 10 and 100 μM) injected 5 times on column and the C.V.s were calculated and found to be 3.8%, 3.0% and 1.2%, respectively, for CySH and 2.5%, 1.5% and 0.5%, respectively, for GSH.

The chromatograms obtained from the analysis in the human samples tested using the mBBr derivatisation procedure (Fig. 1) yielded well separated peaks of adducts between CySH and GSH, with retention times of 4.1 min and 6.2 min, respectively, and the variation of retention times was less than 1% between a multiple of analyses. The concentrations of glutathione were well within the calibration range (from 0.1 to 200 μM) in both unreduced and reduced extract. However, cysteine was undetectable in unreduced extract, whereas in reduced extract it was clearly detectable. To establish the reproducibility of the calibration we did a regression analysis on the mean of 10 independent calibrations for both GSH and CySH. The line equation for GSH was $y = 4.32x$ where y is the integrated area and x the concentration of GSH. Similarly the line equation for CySH was $y = 3.62x$, where x is the concentration CySH. The correlation coefficient for both regressions was $r > 0.998$.

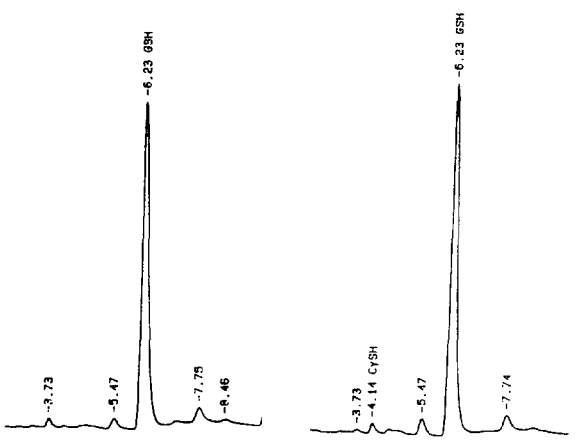

Fig. 1. Typical HPLC traces obtained from the analysis of human skeletal muscle tissue: (A) reduced GSH, (B) total CySH and GSH following reduction of thiols with dithiothreitol (DTT). Retention times are in minutes from the injection point.

3.2. GSH and CySH recoveries from skeletal muscle

Authentic samples of GSH and CySH were added at two concentrations (50 μM and 100 μM) to muscle biopsies before homogenisation. The efficiency of recovery of GSH as bimane adducts is shown in Table 1. The mean recovery of GSH at the lower level was 96.1% and at the higher level was 95.0%. However, we were not able to achieve an equally good recovery of CySH (60–70%) from muscle samples, although the same procedure as for GSH determination was used.

3.3. GSH levels in human skeletal muscle

The concentrations of GSH in skeletal muscle are shown in Table 2. The muscle GSH concentrations in nine healthy young male volunteers were measured in the post-absorptive state and 2 h after food intake throughout the subsequent 24 h. Reduced GSH in muscles ranged from 1000 to 1500 μmol/kg and total GSH ranged from 1200 to 1700 μmol/kg. Statistical analysis showed that there were no significant differences in reduced GSH and total GSH levels between the initial time studied and those obtained at any of the sampling points during the subsequent 24 h of the investigation. As GSSG

Table 1
Recovery of GSH added to skeletal muscle biopsy specimens collected from five individuals

Sample	Basal tissue homogenate concentration (μM)	+ 48.4 μM GSH		Basal tissue homogenate concentration (μM)	+ 94.7 μM GSH	
		Found (μM)	Recovered (%)		Found (μM)	Recovered (%)
1	51.8	102.8	105.4	53.6	146.4	98.0
2	49.9	94.7	92.6	41.0	132.6	96.7
3	47.9	94.0	95.2	32.5	118.3	90.6
4	52.4	98.4	95.0	33.8	124.3	95.6
5	49.4	94.1	92.3	48.1	136.6	93.5
Mean			96.1			95.0

The values given for the 50 μM and 100 μM spiked standards are those obtained by addition of authentic samples. The assay procedure is as described in Section 2.

cannot be determined directly using the present technique, GSH/GSSG ratios were deduced from calculated values of GSSG obtained from the subtraction of reduced GSH from total GSH values. The mean ratio of GSH/GSSG was 13.8 ± 1.1 (S.E.M.). Measurement of GSH concentrations in triplicate muscle biopsy specimens from the same sites of eight individuals yielded coefficients of variation ranging from 2.8 to 6.1%, with a mean value of 4.7 for reduced GSH, and 1.9–6.4 with a mean value of 4.2 for total GSH.

3.4. Concentrations of the constituent amino acids of GSH in muscle

The constituent amino acids for GSH synthesis, glutamate, glycine and CySH, were also followed in the muscle during the 24-h study period. The results of these determinations are shown in Table 3. The CySH values are those determined by derivatisation with monobromobimane. The concentrations of all three constituent amino acids remained unchanged during the period of investigation. It was found that the bimane adduct of CySH was undetectable in muscle tissues, but reduction of the sample with DTT released CySH from soluble disulfides at levels that can be quantitated. This indicates that CySH exists most in oxidised forms in muscle supernatants after sample preparation, probably as cystine or cysteine–GSH mixed disulfides.

4. Discussion

A variety of methods of determining GSH are available [15] but the HPLC-based methods have been found to be the most rapid, sensitive and selective. The method chosen in the present

Table 2
Muscle GSH concentrations of nine healthy young volunteers during a 24-h period

Time (h)	Muscle reduced GSH (μmol/kg wet weight)	Muscle total GSH (μmol/kg wet weight)
08:00	1320 ± 37	1525 ± 61
14:00	1338 ± 38	1577 ± 41
20:00	1349 ± 38	1531 ± 50
08:00	1415 ± 51	1570 ± 41

Values are mean ± S.E.M.

Table 3
Concentrations of GSH constituent amino acids in muscle of nine healthy young volunteers during a 24-h period (values mean ± S.E.M.)

Time (h)	Glycine (mmol/kg wet weight)	Glutamate (mmol/kg wet weight)	Cyst(e)ine[a] (mmol/kg wet weight)
08:00	0.80 ± 0.07	1.73 ± 0.06	0.04 ± 0.03
14:00	0.74 ± 0.09	1.41 ± 0.09	0.03 ± 0.03
20:00	0.59 ± 0.07	1.45 ± 0.16	0.03 ± 0.02
08:00	0.84 ± 0.13	1.52 ± 0.17	0.04 ± 0.03

[a] Values are total CySH concentrations from mBBr-based HPLC analysis.

study employs a one-step derivatisation procedure utilising mBBr for pre-column derivatisation of the reduced thiols. This method also employs DTT for the reduction of thiols in the analysis of oxidised thiol components in biological systems previously used by Cotgreave and Moldéus in rat tissues [5]. Here we have reported a slightly modified procedure coupled with an established technique for taking biopsies from human muscle tissue.

Under standardised conditions, the present HPLC method was sensitive enough (20 pmol per injection) to assay GSH in muscle homogenate samples. The GSH peak was separated to baseline without interference from other cellular thiols in muscle samples (Fig. 1). As reduced GSH is easily oxidised to GSSG, the accurate determination of GSH in biological samples is largely dependent on a suitable treatment of the sample. In the present method, tissue homogenisation, neutralisation of deproteinated homogenate and a derivatisation of the sample were performed as rapidly as possible before storage of the samples. Various acids have been used for deproteinization and the inhibition of GSH oxidation by endogenous enzymes. However, 5-sulphosalicylic acid is suggested to be preferable [16]. Since GSH readily oxidises non-enzymatically at pH values greater than 7, and γ-GT which catalyses the first step of GSH degradation [16] has an optimum activity at neutral pH, the muscle samples were quickly acidified. Under the experimental conditions used in this study, using SSA for extraction, the recoveries of GSH and CySH were evaluated by adding two different concentrations (50 μM and 100 μM) of the thiols to the SSA buffer just before sample preparation. The recoveries of GSH were found to be 96.1% and 95.0%, respectively (Table 1). However, by using the same procedure we were not able to obtain more than 70% recovery of added CySH from muscle extract. We therefore performed an additional experiment on spiked muscle samples, in order to clarify the loss of CySH. Samples spiked with standards were further treated with DTT to reduce oxidised CySH and GSH formed during the sample preparation procedure. We found that 98% of both total CySH and GSH were restored from the acidic extract after the DTT treatment; only about 2% of the determined compounds were lost due to the precipitation of muscle protein. The oxidation of CySH (more than 30% of the reduced form) was much higher than GSH. This could be because CySH is more susceptible to non-enzymatic oxidation than GSH, giving large losses due to the formation of its oxidised form and mixed disulfides during the sample preparation. It is most relevant to determine total cysteine in muscle tissue as cysteine exists at a very low concentration in human tissue due to its toxicity. The high reproducibility (intra-individual C.V.s were less than 5%) of the method when triplicate biopsies were taken from a single individual makes the technique a convenient and reliable tool.

Although skeletal muscle in the rat is the organ, in which the major proportion of total body GSH resides [9], little work has been performed on the GSH status in human skeletal muscle. In this study we have been able to identify GSH as the major thiol pool in healthy humans – since skeletal muscle presents the largest "organ" in human – , as well as to quantitate its concentration, redox status and diurnal variation. The skeletal muscle levels of GSH (1320 ± 37 μmol/kg wet weight) and total GSH (GSH + GSSG, 1525 ± 66 μmol/kg wet weight) in healthy volunteers, obtained by quantitation of mBBr-thiol adducts, were in agreement with those obtained by Corbucci et al. [4] using an enzymatic assay of GSH. A tentative comparison of muscle GSH with data on other tissues obtained by other investigators may be permissible: the cellular concentration of GSH in skeletal muscle was 43% of that in human liver [17], and 20% of that in gastric mucosa [18].

As a major source of thiol in the body, glutathione is a scavenger for reactive oxygen species and free radicals. It also serves as a substrate for the GSH-peroxidases, an important antioxidant enzyme family present in all tissues. Thus, the relatively high GSH content of human muscle tissue may indicate the need for an extensive antioxidant reserve which reflects considerable oxidative metabolism in this tissue. Thus, the calculated values of GSSG in human skeletal muscle cells were higher in proportion to total GSH than those reported from other types of mammalian cells and tissues [2], where it is believed that about 99.5% of intracellular total GSH was in the reduced form. This may reflect a state of continuous, low-level oxidative stress in the tissue, which is associated with a normal physiological function in movement. From the results of our recovery studies, the method itself appeared unlikely to give rise to such high GSSG values. Unfortunately, although monobromobimane prevents artifacts arising from auto-oxidation of glutathione during sample preparation and analysis, it also excludes the possibility to analyse GSH and GSSG simultaneously on HPLC. To accurately differentiate between the two redox forms of GSH, an independent method for the assessment of GSSG is required, such as direct HPLC separation combined with dual electrochemical detection [19], or by initial formation of S-carboxylmethyl derivatives of the free thiols followed by derivatisation of the free amino groups with 2,4-dinitrophenol, followed by HPLC separation with UV detection [20].

To verify the possibility of diurnal variations in GSH levels in skeletal muscle, we took muscle biopsies after overnight fasting and at 2 h after the intake of food in the day time. The results from the healthy male volunteers did not show a significant diurnal variation in skeletal muscle GSH levels during a 24-h period. Under certain circumstances GSH is considered to be a reservoir of cysteine; for this reason we chose to determine cysteine in muscle tissue. It is also of interest to be able to determine the constituent amino acids in the same biopsy specimen, so that relevant comparisons may be made concerning changes in the relationships between GSH and its constituent amino acids.

In conclusion, we report the adaptation of an existing HPLC method using monobromobimane, for the in situ derivatisation of thiols and reliable estimation of the concentration of reduced GSH and total GSH in human skeletal muscle. The method chosen for taking biopsies and biopsy handling enabled us to make accurate and reproducible determinations of both GSH and its constituent amino acids. Moreover, we have determined the normal range of GSH levels in human skeletal muscle, and confirm previous animal studies that this tissue possesses one of the major reservoirs of GSH in the body, reflecting the oxidative potential of its metabolism.

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