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Electrochemical determination of femtomole amounts of free reduced and oxidized glutathione

Application to human hair follicles

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ABSTRACT

A simple and sensitive high-performance liquid chromatographic technique has been developed for the quantification of free reduced and free oxidized glutathione in biological samples. After acidic extraction and isocratic separation of the compounds of interest on a reversed-phase column, both forms of glutathione are quantified with a coulometric detector working in the oxidative mode. The limit of detection is 125 fmol for reduced glutathione and 400 fmol for the oxidized form (signal-to-noise ratio of 3). This sensitivity allows the measurement of the small amount of glutathione present in a single hair follicle. The technique is well adapted to microsamples, *i.e.* for non-invasive sampling technique (hair, skin, tears, etc.) and can be adapted to various cells or tissues.

INTRODUCTION

The tripeptide glutathione (L-γ-glutamyl-L-cysteine-L-glycine) is widely distributed in living cells and is involved in many biological functions [1]. In extra- or intracellular media, glutathione is mainly found as the reduced (GSH) form and, in much smaller amounts, as the oxidized (GSSG) form.

The amount of GSSG in intracellular media may increase with oxidative stress [2] but can be reduced to GSH by the enzyme glutathione reductase. Furthermore, a decrease in glutathione or glutathione enzyme activity with age has been observed in various tissues and organs [3–5], including human skin [6] and hair follicles [7].

Although numerous chemical, enzymatic and high-performance liquid chromatography (HPLC) methods have been reported for the determination of thiols (for a review, see refs. 8 and 9), only a few have described procedures for the simultaneous determination of both oxidized and reduced thiols in a single step or without separation into fractions, or both.

The use of HPLC coupled to electrochemical detection proved to be an attractive solution to a problem such as this. Ordinarily, electrochemical detectors are operated in the amperometric mode, *i.e.*, the detector current is monitored while the working electrode potential is held constant. Typically, between 1 and 5% of the analyte is electrolysed. When the fraction of the sample electrolysed approaches unity, the detector is said to be coulometric.

First proposed by Rabenstein and Saetre [10], the amperometric method was refined by Allison and Shoup [11] and used by others for various studies [12–15]. For the detection of both thiols and disulphides, a dual gold–mercury cell is used in series. After the HPLC separation, the eluate

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passes over a first electrode which acts as a "post-column reactor" and reduces the disulphide to the corresponding thiol, which can then be detected by the catalytic oxidation of mercury on the second electrode. The system is thus capable of detecting both thiols and disulphides in the same sample. However, the method has some disadvantages [16]: (i) a loss of sensitivity of the electrodes, due to "co-oxidized" interfering material, is observed; and (ii) a large amount of thiol must be injected to have a detectable amount of disulphide present. This leads to a fouling of the mercury electrodes and to a rapid decay of the analytical sensitivity [11].

O'Gara *et al.* [17] have quantified GSSG using a coulometric detector operating in the oxidative mode. Their result prompted us to investigate the efficiency of this unusual technique for the simultaneous determination of reduced and oxidized glutathione in hair follicles.

The purpose of this paper is to demonstrate that coulometric detection using oxidative reactions can measure the very low levels of GSSG found in cellular systems under normal conditions and without apparent artifactual oxidation. The uses and limits of the technique are discussed.

EXPERIMENTAL

Reagents

Disodium hydrogenphosphate was obtained from Fluka (Mulhouse, France). GSH and GSSG were from Sigma (St. Louis, MO, USA). Orthophosphoric acid and trichloroacetic acid (TCA) were purchased from Prolabo (Paris, France). All other chemicals were of analytical-reagent grade. Deionized distilled water was used.

Sample preparation

About ten hairs were plucked from the suboccipital area of healthy volunteers (men and women). After examination under a light microscope, three follicles in the anagen phase were selected and homogenized in 75 μl of 5% TCA solution and immediately centrifuged at 5000 *g* for 5 min. A 60-μl aliquot of supernatant were collected for immediate analysis.

DNA assay

The 4,6-diamino-2-phenylindole (DAPI) method described by Meyer and Grundmann [18] was used. The assay was performed with 10-μl aliquots of sample and the DAPI–DNA complex was measured fluorimetrically at excitation wavelength 360 nm and emission wavelength 453 nm.

Determination of glutathione

GSH and GSSG were separated and quantified simultaneously by HPLC and coulometric detection. A 10-μl aliquot of supernatant were injected (Valco valve, Model C6W) onto an Ultrasphere XL-ODS (75 × 4.6 mm I.D.; 3 μm particle size) obtained from Beckmann France (Gagny, France). A precolumn, packed with the same material, was used to increase the column lifetime. The mobile phase (50 m*M* sodium phosphate adjusted to pH 3 with phosphoric acid) was run at a flow-rate of 0.5 ml/min. Before use, the mobile phase was filtered through a 0.22-μm anodic membrane from Anotec (Banbury, UK).

The coulometric detector (Model 5100 A with Model 5011 analytical cell) from ESA (Bedford, MA, USA) contained two porous graphite electrodes (E_1 and E_2) in series. A guard cell held at +0.9 V *versus* Pd, between the pump and the injector, was used to minimize the background current. The upstream electrode (E_1), held at +0.73 V *versus* Pd, was used for the detection of GSH. Oxidized glutathione was quantified with the downstream electrode (E_2) held at +0.86 V *versus* Pd.

Electronic signals from the electrodes were monitored by use of a two-pen strip-chart recorder from Knauer (Bad Homburg, Germany). Quantification was made by peak height measurements using external standards prepared daily.

RESULTS

Detection

For the quantification of the compounds of in-

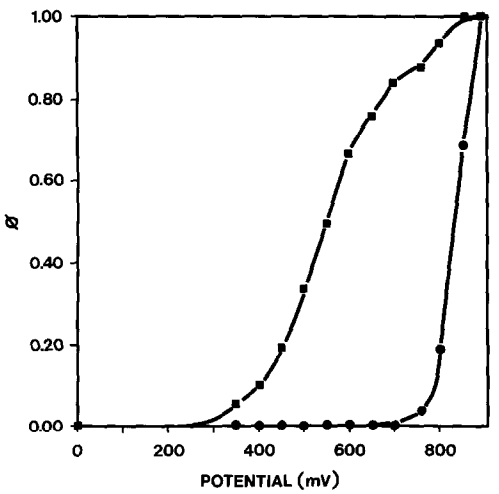

Fig. 1. Hydrodynamic voltammograms of GSH (■) and GSSG (●) on the upstream electrode. Potential of the downstream electrode is held at +0.89 V. ϕ is the ratio of the peak current measured for a given voltage to the peak current measured at +0.89 V. Values plotted are the mean of triplicate injections.

terest, we investigated the electrochemistry of GSH and GSSG by generating hydrodynamic voltammograms (current *versus* voltage curves). For this assay, the potential of the downstream electrode was held at +0.89 V whereas the potential of the upstream electrode was varied from +0.35 to +0.89 V by 0.05 V steps. A response

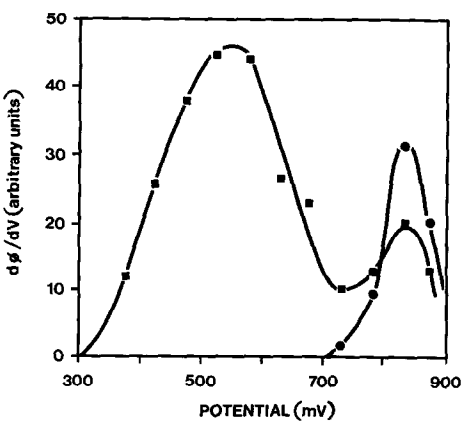

Fig. 2. First derivative ($d\phi/dV$) of the hydrodynamic voltammograms. Two peaks are observed for GSH (the first peak corresponds to a potential $V_1^* = 0.55$ V and the second to a potential $V_2^* = 0.83$ V). Only the second peak is observed for GSSG. ■ = GSH; ● = GSSG.

due to the oxidation of GSH was observed for a potential of more than +0.3 V (see Fig. 1). For GSSG, the threshold potential was in the range 0.70–0.75. A biphasic behaviour of the response curve corresponding to GSH was observed. Just before the electrochemical response reached saturation it showed a further voltage-dependent increase. This phenomenon is depicted in Fig. 2, which represents the first derivative of the current *versus* voltage curve for both compounds. The first peak, only observed for reduced glutathione, could be assigned to the oxidation of the thiol group; the second, observed for both compounds, was attributable to the oxidation of the amine group(s).

Therefore, by holding E_1 and E_2 at 0.73 and 0.86 V, respectively, 90% of the overall signal corresponding to GSH was detected with the upstream electrode. GSSG was only detected with the downstream electrode.

Peak assignment

The comparison between currents (A_1 and A_2) from the upstream and the downstream electrodes, respectively, was used to control the peak purity for the compounds of interest and for the detection of co-eluting substances by comparing the A_1/A_2 ratio to the values obtained with authentic standards. Furthermore, peaks are assigned by comparing their capacity factors with those of the pure compounds.

Limit of detection

A linear relationship between the amount injected and the detector response, over at least three decades, was observed for both compounds ($r > 0.999$). From the experiments, we concluded that the limits of detection (signal-to-noise ratio of 3) for injected GSH and GSSG were 125 and 400 fmol, respectively.

Reproducibility

The potentials of the electrodes were usually set in the range giving the maximum current response for a given amount of compound. This was not so in our experiment to avoid co-oxidation of interfering material. However, the repro-

TABLE I

REPRODUCIBILITY OF GSH AND GSSG PEAK HEIGHTS

125 pmol GSH and GSSG injected.

Injection No.	Peak height (arbitrary units)	
	GSH	GSSG
1	97	16.8
2	98	17.1
3	98	17.2
4	98	17.0
Mean value	97.75	17.03
Standard deviation	0.5	0.17
Coefficient of variation (%)	0.51	1.0

ducibility appeared to be satisfactory (Table I) as the relative deviation is in the 1% range with standard solutions.

Recovery studies

Estimation of the recovery of added GSH and GSSG was ascertained by addition of known amounts of these substances to the homogenate (ten hair follicles in 350 μl of the 5% TCA solution). Three homogenate mixtures were prepared: hair follicles alone (no added GSH or GSSG), hair follicles plus 2 nmol of GSH and hair follicles plus 0.05 or 1 nmol of GSSG. The different experiments (see Table II) yielded acceptable mean recovery values (102 ± 6% for GSH and 105 ± 14% for GSSG). Furthermore, it can be concluded from Table II that the difference between the measurements of the spiked native sample and the native sample alone yielded approximately the concentration value of added compound. This observation demonstrated that no significant reduction or oxidation process occurred during the extraction step.

TABLE II

RECOVERY STUDIES OF GSH AND GSSG ADDED TO HAIR FOLLICLE SAMPLES

Values indicate mean ± standard deviation for the number of determinations shown in parentheses. Initial amounts for GSH and GSSG are 2 nmol and 30 pmol, respectively.

Experiment No.	Compound added	Amount added (nmol)	Recovery (%) (mean ± S.D.)
1	GSH	10	99 ± 4 (6)
	GSSG	0	116 ± 38 (6)
2	GSH	0	104 ± 7 (6)
	GSSG	0.05	99 ± 2 (3)
	GSSG	1.0	98 ± 2 (3)

The instability of GSH, due to its oxidation to GSSG on exposure to air, had been studied by investigating the influence of the delay in time between homogenization and sample determination. Samples prepared and stored under acidic conditions, *i.e.* homogenization with 5% TCA, showed a loss of *ca.* 1% for GSH after 1 h at +4°C, but no change for GSSG. We suggest, therefore, that samples should be measured as soon as possible after homogenization.

Application to hair follicles

To test the sensitivity of the technique, GSH and GSSG were determined in a single hair follicle. Reduced and oxidized glutathione were easily detected (Table III). GSH was quantified with the upstream electrode (Fig. 3), GSSG with the downstream electrode (Fig. 4). The comparison of signals from both electrodes indicated negligible interference with co-eluted compounds as peak ratios and capacity factors for GSH and GSSG were in agreement with standard assays.

DISCUSSION

Generally, when using amperometric detection for the determination of GSSG, oxidized glutathione was first reduced to the thiol and then quantified by subsequent oxidation of the thiol group to the corresponding disulphide. Such a procedure had been developed, in our opinion, for the following reasons.

Although the functional amine group(s) of GSSG can be oxidized on a carbon surface, this oxidation requires high potentials (over 1.0 V *versus* Ag/AgCl). Under such conditions, detection is no longer specific and makes direct detection of GSSG intractable for trace determina-

TABLE III

CONCENTRATIONS OF DIFFERENT GLUTATHIONE FORMS IN HAIR FOLLICLES OBTAINED FROM HEALTHY VOLUNTEERS

Subject No.	Sex	Mass of DNA per follicle (μg)	GSH (nmol/μg DNA)	GSSG (nmol/μg DNA)	GSH/(GSH + GSSG) (%)
1	Male	0.13	5.53	0.053	0.95
2	Female	0.055	14.1	0.110	0.77
3	Male	0.045	5.11	0.041	0.80
4	Male	0.028	3.07	0.033	1.06
5	Female	0.193	3.68	0.080	2.13
6	Female	0.108	6.20	0.280	4.32
7	Female	0.032	4.06	0.178	4.20
8	Female	0.064	1.87	0.072	3.71
9	Male	0.064	1.25	0.009	0.72
10	Male	0.062	5.64	0.100	1.74
Mean value		0.078	5.05	0.096	2.04
Standard deviation		0.051	3.58	0.080	1.48
Coefficient of variation (%)		65.4	70.9	81.6	72.69

tions as the background current is very high. With a mercury–gold electrode, the indirect detector reaction is based on the oxidation of mercury in the presence of thiols and requires very low potentials, typically 0.1 V *versus* Ag/AgCl, in contrast to the 0.9–1.0 V potentials used for the direct oxidation of thiol to the disulphide on a carbon surface. Owing to this low potential, the

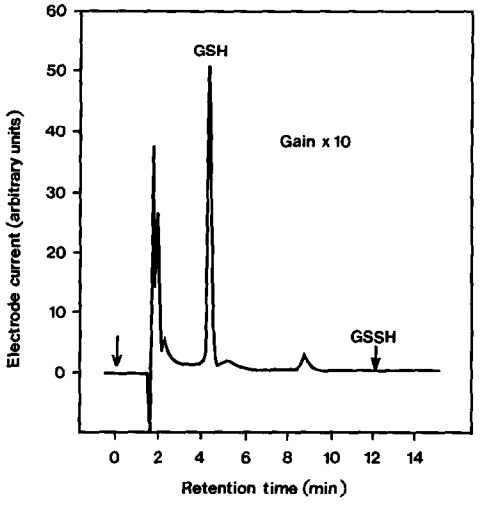

Fig. 3. Response of the upstream electrode (+0.73 V) to a hair follicle sample (62.5 pmol of GSH are injected, *i.e.*, 0.5 nmol per follicle). GSSG is not detected with this electrode (for chromatographic conditions, see text).

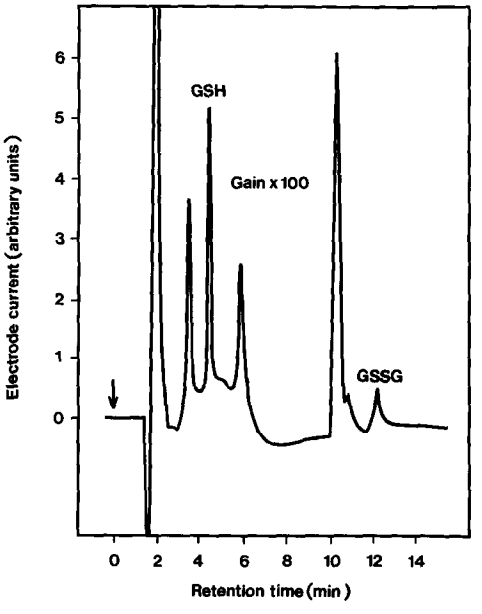

Fig. 4. Response of the downstream electrode (+0.86 V) to a hair follicle sample. A signal response due to GSH and GSSG is observed. For the detection of oxidized glutathione (1.9 pmol of GSSG injected, *i.e.*, 11 pmol per follicle), the sensitivity has to be increased by a factor of ten in comparison with the upstream electrode (for chromatographic conditions, see text).

detection is extremely specific: only sulphydryls, halide or similar ions and chelating agents can be detected.

However, a different approach to the problem was also possible. Usually, electrochemical detectors are operated in the amperometric mode, *i.e.*, between 1 and 5% of the analyte is electrolysed. With a coulometric detector, about 100% of the analyte is electrolysed. It is thus possible to achieve a quasi-complete electrolysis of the eluate using the trapping capabilities of a coulometric guard electrode. As the background current is mainly due to electrochemically active trace constituents in the mobile phase, a coulometric guard cell, with a large porous electrode, can be used to "remove" these impurities before the injector so that the analytical cell receives only electrochemically "clean" mobile phase and the background current is shut down to an acceptable level.

From an electrochemical point of view, the detector specificity is not efficient enough when using high positive potentials, as many functional groups are then oxidizable. To partially overcome this problem, we used a technique similar to the simultaneous monitoring of two wavelengths of a UV detector. Absorbance ratios are normally used to provide an indication of the peak purity by comparing the observed ratios to those obtained with authentic standards. In our experiments we compared the current from both the upstream (A_1) and the downstream electrode (A_2). About 90% of the total current due to GSH was detectable with the upstream electrode. Therefore the ratio A_1/A_2 remained constant and was concentration independent. GSSG was only detectable at the downstream electrode because the potential of the upstream electrode was below the threshold required for the oxidation of its amino group. The appearance of the GSH signal in the chromatogram at a lower retention time (see Fig. 4) and due to the remaining amount of about 10% of non-oxidized GSH (see above) did not impair the determination of GSSG. This is a very important fact because GSSG represents only a small fraction compared with the concentration of GSH. Although only about 10% of GSH was accessible to oxidation at the downstream electrode it could be measured with a high sensitivity. None, or at least only minor, interferences were observed. Assigment of peaks for either GSH or GSSG was achieved by comparing the retention times of sample peaks with those of the authentic compounds. Thus by the use of two electrodes held at a different potential we were able to detect both oxidized and reduced glutathione. The main advantage of the "classical" configuration consisted in the fact that possible interferences were removed by the "low potential" upstream electrode. Subsequent measurement of the analyte took place only at the "high potential" downstream electrode.

Minimal detectable amounts (signal-to-noise ratio of 3) are 0.125 pmol for GSH and 0.4 pmol for GSSG. These values are significantly lower than those reported by Allison and Shoup of 3.5 and 5.7 pmol for GSH and GSSG, respectively [11]. This result emphasizes the advantages of a coulometric detection *versus* amperometric or fluorometric detection [19,20]. Optimization of the sensitivity of the method also depends on the chromatographic parameters. Microbore packed columns (1–2 mm I.D.) [21,22] or short columns packed with 3 μm particles [23,24] are well designed for this purpose. In this study only 3-μm particle columns were tested. Both theory and experiment have shown the increase in efficiency which can be obtained by decreasing particle size. From a practical point of view, higher efficiency provides improved resolution, shortened analysis time and (for a given sample mass) increased sensitivity. It was only necessary to minimize extra-column band broadening [25], especially for early eluting peaks (low capacity factor) which have small volumes [24]. The coulometric detector, with a cell volume less than 5 μl, allowed the use of such a column. Therefore the overall sensitivity of the analytical system was high enough to quantify the amount of GSH and GSSG in one hair follicle. Mean values for GSH and GSSG, obtained from ten healthy volunteers, were 5.05 nmol/μg DNA and 0.096 pmol/μg DNA, respectively. However, wide variations were observed for both compounds. Similar results were ob-

tained by Pruche *et al.* [26] who have quantified total glutathione (GSH + GSSG) in human hair follicles (n = 43 volunteers under 80 years of age) according to the enzymatic method of Griffith [27]. The total glutathione content was 5.42 ± 3.94 nmol/μg DNA (mean value ± standard deviation). In their study, the variations observed decreased as a function of age. The "young" population (under 80 years of age) shows a greater variation in comparison with the older population (from 80 to 103 years).

CONCLUSION

When using the trapping capabilities of a coulometric detector, quantification of GSSG is possible, through oxidative reactions involving the amine group(s) of this compound, as the background level due to the mobile phase is reduced to an acceptable level. The simplicity of the technique, which requires neither a rigorous deoxygenation of the mobile phase nor a frequent cleaning of the electrodes, makes it readily applicable to routine analysis. Investigations are in progress towards the determination of glutathione in various biological matrices (tears, skin, etc.).

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