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Determination of Thiol Concentrations in Haemolysate by Resonance Raman Spectrometry

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A simple and sensitive method for determining the thiol concentration in haemolysate, using the resonance Raman spectrum of the product of the reaction of the lysate with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), is described. The method uses a signal due to the haem ring of haemoglobin as an internal calibrant. In six separate determinations of the thiol concentrations in the lysate from a normal male volunteer, the average thiol concentration was 1820 μM and the relative standard deviation was 4.5%. Standard addition of glutathione to four lysate samples confirmed this result and indicated an acceptable precision. To obtain a comparison with another technique, clear lysates were prepared by precipitation of haemoglobin and addition of sufficient glutathione to maintain thiol levels comparable to those of coloured lysates. Agreement between the resonance Raman method and a spectrophotometric method was acceptable in this instance. The method is relatively free from the standard interferences found for spectrophotometric assays and is selective. The mean value for the lysate from six normal male volunteers (1920 ± 88 μM) and from seven patients with rheumatoid arthritis (3240 ± 774 μM) were significantly different, suggesting that this measurement may have relevance in the study of disease processes.

Keywords: *Thiol determination*; *haemolysate*; *resonance Raman spectrometry*

Thiol concentrations within cells are seldom determined in clinical studies, in spite of the fact that they are believed to play a key role in vital processes such as transport, protein synthesis and membrane stabilisation.[1–3] One reason for this is that present methods of determination are usually based on spectrophotometric methods[4,5] and are therefore more suited to measurements in colourless plasma than in lysates that contain haemoglobin. A second reason is that, although some evidence of thiol level changes in disease states is available, most measurements have been concerned with plasma thiol levels. With intracellular thiols, an oxidation to the disulphide tends to occur during the separation process, making the measurements of questionable value. One method used to overcome this, the reaction of SH groups with *N*-ethylmaleimide[5] to produce a compound with an absorption band, which can be used for the determination of thiol in the presence of haemoglobin, probably causes partial protein denaturation.[6]

In this paper we report a reliable, selective and sensitive technique based on resonance Raman spectrometry, which requires very simple and relatively mild separation techniques. The method has been used to indicate that, in one disease at least (rheumatoid arthritis), the change in thiol status in the lysate from packed blood cells is considerable.

Experimental

Materials

Stock solutions of 10^{-2} M 5,5′-dithiobis(2-nitrobenzoic acid) (BDH Chemicals) (Ellman's reagent) and 2.23×10^{-2} M glutathione (Sigma) were prepared in a 10^{-1} M disodium hydrogen orthophosphate buffer solution adjusted to pH 7.6 with hydrochloric acid. De-ionised water was used throughout.

Methods

Samples of 5 ml of human blood treated with heparin to prevent clotting were centrifuged in

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a Mistral 6L centrifuge at 4000 rev min^{-1} for 10 min. The plasma was removed with a Pasteur pipette and washed three times with 0.9% saline solution and the cellular fraction was lysed with an equal volume of distilled water for 1 h. The lysed cells were re-centrifuged at 4000 rev min^{-1} for 10 min and a sample of the lysate was removed from the tube with a pipette. A 0.1-ml volume of the lysate diluted with 3 ml of 10^{-1} M disodium hydrogen orthophosphate buffer was placed in a 1-cm glass fluorescence cell, and 0.5 ml of 10^{-2} M Ellman's reagent stock solution was added.

The cell was placed in the sample compartment of a Cary 81 Raman spectrometer modified by Anaspec Ltd. and equipped with a cooled photomultiplier and photon counter. It was irradiated with 100 mW of laser light at 457.9 nm from a Spectra-Physics 17–19 argon ion laser; 90° Raman scattering between 1200 and 1400 cm^{-1} from the exciting line was recorded.

In the standard additions experiments, successive additions of 5 μl of the 2.23×10^{-2} M glutathione stock solution were added to solutions containing 0.2 ml of haemolysate and 0.5 ml of the stock solution of 10^{-2} M Ellman's reagent, in 5-ml calibrated flasks and the contents were made up to the mark with 0.1 M disodium hydrogen orthophosphate buffer adjusted to pH 7.6 with hydrochloric acid.

By this method, addition of approximately 0.5, 1.0, 1.5, 2.0 and 2.5 thiol groups per haemoglobin molecule was obtained. Precise values were determined for each experiment on the basis of the haematocrit.

Clear lysate was prepared using a standard chloroform - ethanol precipitation technique.[7] Before measurement, 200 μl of 500 μM glutathione solution were added to 1.5-ml aliquots of the clear lysate to produce solutions with thiol concentrations of the same order of magnitude as that found in haemolysate.

Throughout this paper, thiol levels are expressed per millilitre of the haemolysate used for measurement. They are therefore half of the value expected for undiluted cytosolic fluid.

Results and Discussion

The most commonly used method for the determination of thiol concentrations in clinical studies is Ellman's method,[4] which utilises an exchange reaction between the thiol and 5,5′-dithiobis(2-nitrobenzoic acid) (ESSE):

$$\mathrm{ESSE} + \mathrm{RSH} \rightleftharpoons \mathrm{ESSR} + \mathrm{ESH} \quad \ldots \quad \ldots \quad \ldots \quad (1)$$

At the neutral pH employed in the determination, ESH is ionised to ES^-. This is a coloured species with a band maximum at 412 nm, which can be used to determine the thiol concentration. This method is sensitive to interferences. For example, it is not possible to use it directly in the presence of haemoglobin and a technique in which the thiol is made to react with *N*-ethylmaleimide has to be adopted. Further, different thiols react in different ways with ESSE when they are present in equimolar amounts or in excess.[7] For example, some thiols remain as the mixed disulphide, ESSR; others react further to give RSSR and liberate a second ESH molecule.

Therefore, the common practice of using excess of ESSE in the analysis is necessary. If this excess is too large, the band edge of the ESSE peak at 325 nm interferes with the determination of the ES^- concentration. Consequently, a carefully balanced reference solution is required, which presupposes a knowledge of the approximate thiol concentration to be measured.

Glutathione in solution has no detectable Raman spectrum at a concentration of 1.0 M under the conditions described in the previous section. A 1 + 1 mixture of 10^{-4} M glutathione and ESSE gave a strong Raman signal consisting of two bands at 1325 and 850 cm^{-1}, which have previously been assigned to a symmetrical stretch and an in-plane bending mode of the nitro group.[8,9] A resonance profile of the band using the available laser lines indicates that the enhancement is likely to be due to the 412-nm band (Fig. 1). The spectrum of ESSE is, as might be expected, much weaker and the most intense band, the A_1 symmetrical stretch, is shifted to 1348 cm^{-1}. Hence, this mode can be used to differentiate ES^- from ESSE and can form the basis for a sensitive analytical technique that should be specific for ES^-.

A major problem with the use of a Raman spectrometer for quantitative analysis is that it is inherently a single-beam technique, although special divided rotating cells have been described.[10] With the present method, when the above experiments were repeated with haemolysate in place of glutathione, a peak due to resonance from haemoglobin was observed at 1373 cm^{-1} (Fig. 2) and unreacted Ellman's reagent could only be detected when present in considerable

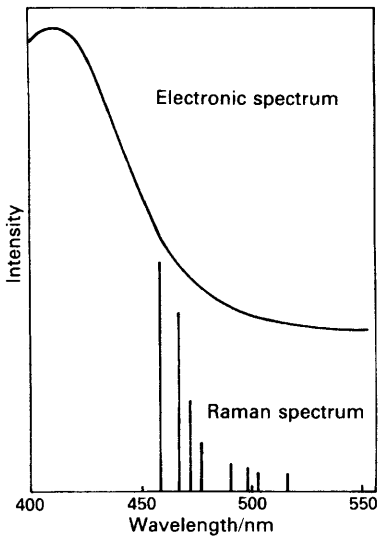

Fig. 1. Resonance Raman profile for the reaction of Ellman's reagent with glutathione. Band intensities refer to the 1325 cm^{-1} band and increase with increasing excitation energy.

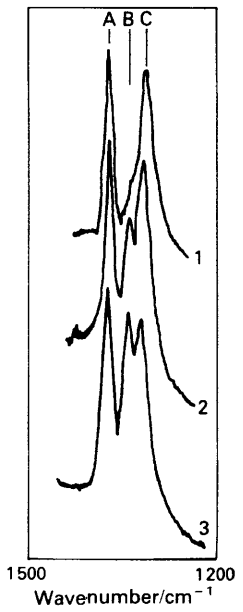

Fig. 2. Resonance Raman spectrum of the products of the reaction of Ellman's reagent (ESSE) with haemolysate. In spectrum 1 a 5-fold excess, in spectrum 2 a 20-fold excess and in spectrum 3 a 100-fold excess of ESSE were used. Peak A is due to haemoglobin, peak B to unreacted ESSE and peak C to ES^-.

excess. As haemoglobin concentrations are routinely determined in most clinical analyses of blood, it was decided to use this peak as an internal standard and to express the thiol concentration per haemoglobin molecule. This means that the absolute determination of thiol concentration is dependent not only on the thiol assay but also on the accuracy of the haemoglobin determination. However, haemoglobin itself contains thiol groups and there is a link between haemoglobin metabolism and thiols.[11] The present method will therefore be particularly appropriate in studies of this relationship, as the relative concentrations are measured in the same solution.

To determine the thiol concentration, standard additions of glutathione were made to the haemolysate. A graph of the ratio of the peak area of the thiol peak to that of haemoglobin against the concentration of added thiol enabled the thiol concentration per haemoglobin molecule to be calculated (Fig. 3). In many instances, intracellular parameters have been expressed in this way but, if an absolute thiol measurement is required, this can be obtained from a knowledge of the haemoglobin concentration in the blood, which is a routine measurement in most hospitals. Standard addition graphs corrected for changes in the haemoglobin concentration have similar slopes for both normal and rheumatoid lysate and indicate that there is an acceptable precision (Fig. 4). Further, the thiol concentration per unit area of the haemoglobin peak is a constant and, consequently, it is possible to calculate thiol concentrations from single measurements by this method and also by the use of standards. Repeated measurements of the haemolysate from the blood of one normal male volunteer gave a thiol content of 0.65 thiol groups per haemoglobin molecule (thiol concentration 1820 μM; relative standard deviation = 4.5%; detection limit = 8.1×10^{-5} M).

Few problems result from the usual causes of interference. There was no appreciable effect on the signal in a glutathione solution with ESSE when the sodium chloride concentration was increased from zero to that of physiological saline. A pH range of 5.5–8.5 was investigated and there was little effect on the signal. It seems that any pH at which ESH is ionised to

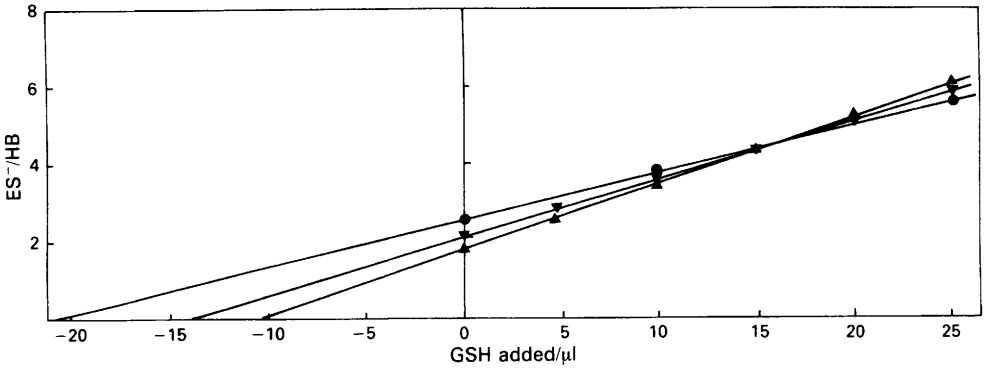

Fig. 3. Standard additions of glutathione to haemolysate. The vertical axis is the thiol to haemoglobin molar ratio and therefore the slope of the line is dependent on haemoglobin concentration.

ES^- is suitable. It is unlikely that any protein would have a resonant signal exactly at 1325 cm^{-1} and there is no evidence of this in dilution studies. However, Raman measurement can be affected by a broad-band fluorescence, which raises the base line and decreases the sensitivity of the signal. We observed this effect in plasma, and the ultraviolet - visible technique discussed earlier is probably preferable in that instance, although special methods have been developed to deal with the fluorescence problem in Raman spectrometry and these might, if available, overcome this disadvantage.

Thiol groups are sensitive to oxidation and in the cell they are involved in the reduction of oxygen.[12] Therefore, one problem with their determination is to ensure that the redox balance present in the intact cell is as little disturbed as possible. For example, the determination of the thiol content of a normal erythrocyte using *N*-ethylmaleimide[5] to prevent thiol oxidation indicated that there was a lysate thiol concentration of about 7300 μM, with 5800 μM resulting from haemoglobin and about 1500 μM from glutathione. In cell lysate produced at neutral pH and from which the haemoglobin was precipitated by a standard chloroform - ethanol procedure, thiol levels of 50–1008 μM were recorded using Ellman's method.

N-Ethylmaleimide will partially denature haemoglobin and it is likely that thiols on haemoglobin are not so readily available as these results might suggest. For example, cystine will react with only one thiol group per haemoglobin,[13] as opposed to two for *N*-ethylmaleimide, and a wide range of other disulphides that also contain a carboxylic acid group will not react at all.[13] Ellman's reagent is a reagent of the latter type. The resonance Raman method indicates a lysate thiol concentration of 1920 ± 88 μM in the blood of seven normal male volunteers (Table I). It seems likely that this is a measure of the non-haemoglobin thiol content of the red cells and that it is not appreciably lowered by oxidation.

In order to obtain a comparison of the method with another technique, and in view of our reservations with regard to the use of *N*-ethylmaleimide, lysates from which the haemoglobin had been precipitated were treated with glutathione to restore the thiol levels to approximately those found in the coloured lysate. This clear solution is suitable for measurement by the

TABLE I

COMPARISON OF THIOL CONCENTRATIONS IN THE LYSATE OF THE BLOOD OF NORMAL VOLUNTEERS WITH THAT OF PATIENTS WITH RHEUMATOID ARTHRITIS

Thiol concentration/mM	
Normal	Rheumatoid
1.84	2.81
1.92	4.06
1.94	3.62
1.92	3.27
1.85	3.27
1.91	3.76
2.07	—

standard spectrophotometric method described by Ellman.[4] No internal reference is possible for the Raman technique, which consequently is liable to the problems inherent in a single-beam method. However, a calibration graph constructed using glutathione indicates that an acceptable accuracy can be obtained (Fig. 5). Cysteine and penicillamine give similar calibration graphs suggesting that the method is not thiol dependent. In a set of five determinations with lysates from different persons, reasonable agreement between the spectrophotometric assay and the Raman assay was obtained ($r = 0.94$; $p < 0.001$).

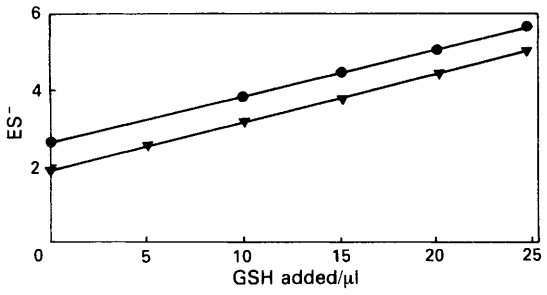

Fig. 4. Standard additions of glutathione to blood cell lysates. Thiol concentrations were calculated from plots similar to those in Fig. 3 and the graph was corrected for changes in haemoglobin concentration.

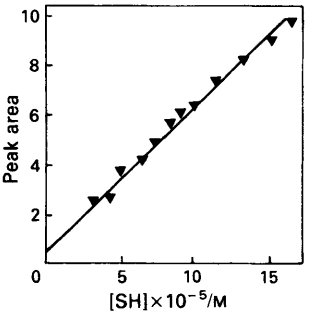

Fig. 5. Calibration graph for glutathione.

A comparison of the thiol concentrations in the coloured lysate from normal and rheumatoid patients is shown in Table I. This indicates a much greater thiol content per haemoglobin with a greater variation between subjects (3240 ± 774 μM). The implications of these results for the study of disease states have yet to be evaluated. However, there is a prodigious literature on non-haemoglobin thiols that suggests that one major fraction is glutathione and that this glutathione level is of considerable importance in cell metabolism, and the haemoglobin thiol status may be of use in studies of the anaemia associated with rheumatoid arthritis.

In conclusion, the resonance Raman method is capable of the determination of thiol concentrations in haemolysate in a simple, reliable and accurate way. It has one particular advantage in that it is selective and it may be possible to analyse for different species with the same sample by the addition of reagents that produce other coloured species. Further, the beam size is small and surface Raman studies are feasible, so that it may be that considerable development of this method is possible, with one of the main limiting factors being, as it often is in Raman spectrometry, the problem of fluorescence.

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