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USE OF ISOTHERMAL MICROCALORIMETRY FOR PREDICTION OF OXIDATIVE STABILITY OF SEVERAL AMINO ACIDS

R. Roškar[*]*, M. Vivoda and V. Kmetec*

Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, 1000 Ljubljana, Slovenia

Isothermal microcalorimetry has been applied as a method for predicting (in)stability of ascorbic acid and several amino acids that undergo oxidative degradation in aqueous media. The fast and simple method involved the addition of different amounts of hydrogen peroxide. The appearance of the heat flow curves gave a clear general indication of how stability was influenced. The accuracy of the microcalorimetric result was investigated by comparing it with an HPLC assay and a good agreement between the results of both methods was demonstrated. It was also established that susceptibility to oxidative degradation decreases in the following order: cysteine, methionine, ascorbic acid, tyrosine and tryptophan.

Keywords: *amino acids, ascorbic acid, HPLC, isothermal microcalorimetry, oxidation*

Introduction

Calorimetry has found applications in a wide range of disciplines within academic science and industry due to the fact that heat is associated with virtually all processes in nature. The potential range of applications of isothermal microcalorimetry in the pharmaceutical sciences is extremely large, but it has not been widely used, possibly due to the inherent lack of specificity, instrument costs, advances in other techniques and regulatory considerations. The greatest promise for microcalorimetry lies in measurements of very complex systems where a single specific technique would not be applicable [1]. The most promising pharmaceutical applications have been reviewed [1–5].

Stability testing is very important during the development of drug products. The use of microcalorimetry in this field offers much potential, but there is also a need to balance good experimental design with careful interpretation. Investigation of physical [6–9] and chemical stability of pharmaceuticals [10–15] by this technique can therefore be of great importance. Many drugs have been subjected to microcalorimetric investigation, including, for instance, ampicillin [10], ascorbic acid [11–12], diclofenac [13], enalapril [14], nevirapine and azidothymidine [15].

In the pharmaceutical field, oxidation is one of the most common decomposition mechanisms studied for drug compounds. The oxidation reactions usually involve complex pathways with multiple intermediates and products [16]. The mechanisms behind these reactions are therefore often poorly understood, making isothermal microcalorimetry a suitable analytical tool for studying these complex processes. Oxidation is one of the major chemical degradation pathways for protein pharmaceuticals as well, where the side chains of methionine, cysteine, histidine, tyrosine and tryptophan residues are potential oxidation sites [17, 18]. Complex protein degradation, which can involve other decomposition pathways, can result in loss of biological activity, undesirable immunogenicity, and altered pharmacokinetics [19]. An indication of any changes in the early development phases of such pharmaceuticals is therefore of great importance.

The objective of our study was to evaluate the microcalorimetric technique for monitoring oxidation reactions of ascorbic acid and several oxidation sensitive amino acids in aqueous solution at 25°C. The heat flow responses were investigated by testing the impact of an oxidant (hydrogen peroxide) on the decomposition of selected compounds. The results were then compared to those obtained by an HPLC assay that measures the amount of the unchanged compound remaining in the solution.

Experimental

Materials

L-ascorbic acid and amino acids *L*-methionine, *L*-cysteine, *L*-tyrosine and *L*-tryptophan were obtained from Sigma (USA). The purity of all compounds was greater than 98%. Hydrogen peroxide

* Author for correspondence:

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Akadémiai Kiadó, Budapest, Hungary
Springer, Dordrecht, The Netherlands

30% was purchased from Fluka (Switzerland). For the HPLC assays, acetonitrile (Merck, Germany, HPLC grade) and orthophosphoric acid 85% (Merck, Germany, p.a.) were used.

Preparation of sample solutions

Compounds for testing were dissolved in water to give the concentrations of 0.5 g L^{-1} (cysteine, methionine and ascorbic acid) and 0.05 g L^{-1} (tyrosine and tryptophan). The final sample solutions in 0.3, 0.03 and 0.003% hydrogen peroxide were obtained by adding 30% H_2O_2 to the prepared compound solutions. Control water solutions with the same concentration of compound as in the sample solutions were prepared to obtain the starting 100% content value, because of the fast decomposition in the presence of hydrogen peroxide.

Methods

Isothermal microcalorimetry

A MicroDSC III (Setaram, France) calorimeter, operating in the isothermal mode at $25\pm1\cdot10^{-4}$ °C, was used together with Hastelloy closed batch vessels. 850 μL of sample solution was loaded into the sample vessel immediately after solution preparation. The reference vessel was filled with water (850 μL). The instrument was operated following the manufacturer's instructions. Baseline drift was within Setaram specification limits when both cells were loaded with water. A 5 min equilibration time was allowed before data were collected using the built in Setsoft software package. In all experiments the heat flow signal was recorded for 20 h.

HPLC assay

The sample solutions prepared for the microcalorimetric measurements were analyzed by HPLC immediately after preparation to obtain a 100% content value for the tested compounds. Directly after the termination of microcalorimetric measurement, HPLC analyses were performed simultaneously on the sample solution from the microcalorimetric vessel and on the same solution stored during the experiment in a flask at 25°C. All analyses were made in duplicate. The HPLC analyses were carried out using a HP 1100 high performance liquid chromatograph controlled by Chemstation, equipped with thermostated autosampler, column heater and diode array detector (Agilent, USA). All HPLC assays performed in this study exhibited assay accuracy (relative error) and precision (relative standard deviation) better than ±1%. The HPLC column was Synergy Hydro (250x4.6 mm, 4 μm) with guard column (4×3 mm) supplied by Phenomenex (USA). The mobile phases were mixtures of 0.01% phosphoric acid and acetonitrile used at a flow rate of 1 mL min^{-1}. Amino acids were detected at their absorption maxima. The HPLC analyses of the compounds tested were not influenced by the presence of hydrogen peroxide.

Results and discussion

The sample solutions of tested compounds for the microcalorimetric measurements were prepared by addition of different concentrations of hydrogen peroxide. The power–time curves for solutions of ascorbic acid exposed isothermally for 20 h at 25°C are presented in Fig. 1. Ascorbic acid exhibits a high starting heat flow for all solutions containing H_2O_2. This then decreases, with steeper curves at higher oxidant concentrations. Hydrogen peroxide did not influence the heat flow signal of tested solutions (Fig. 1 insert). Chromatographic analyses confirmed rapid oxidation in the presence of hydrogen peroxide. After 20 h the decomposition of ascorbic acid was complete in 0.3% and 0.03% H_2O_2 solution; about 15% of the initial amount was still present in 0.003% solution. The extent of ascorbic acid degradation in solutions (a), (b) and (c), stored for 4 h in flasks at 25°C, was 94, 63 and 23%, respectively. Thus, good agreement was obtained between the profiles of heat flow curves and data obtained by HPLC, which is also in accordance with similar experiments performed on ascorbic acid [11].

Similar experiments were carried out to examine the stability of the oxidation sensitive amino acids methionine, cysteine, tyrosine and tryptophan. Methionine, which is known to be the amino acid residue most prone to oxidative deterioration in protein

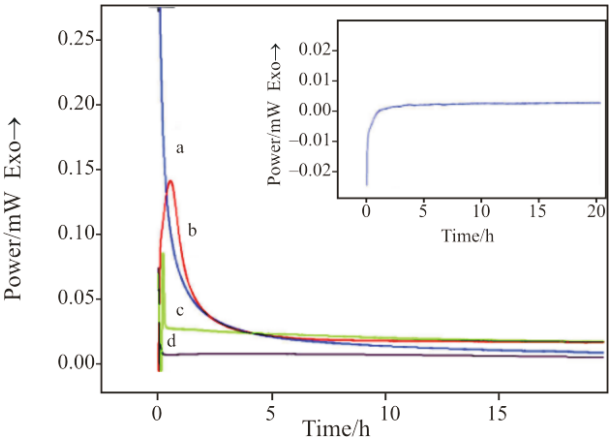

Fig. 1 Power–time curves for the oxidation of ascorbic acid in a – 0.3% H_2O_2, b – 0.03% H_2O_2, c – 0.003% H_2O_2, d – water solution. The insert shows heat flow curve of 0.3% H_2O_2 solution without added ascorbic acid

pharmaceuticals [18], showed a similar pattern to those obtained for ascorbic acid, which indicates a fast rate of oxidation (Fig. 2). The rate of methionine decomposition increases with the concentration of oxidant. Here again, these results were consistent with the fractions of oxidized amino acid obtained by HPLC: 100, 97, 24 and 0.1% for 0.3, 0.03 and 0.003% H_2O_2 solution and water solution, respectively.

Cysteine was also observed to be highly sensitive to oxidation by hydrogen peroxide. This was confirmed by chromatographic analyses performed after 20 h, showing that only in the case of 0.003% H_2O_2 solution was the cysteine decomposition not complete. For 0.3 and 0.03% H_2O_2 solutions stored in flasks, the reaction was complete after approximately 0.25 and 2 h, respectively. We established also in the case of cysteine oxidation a good positive correlation between the oxidant concentrations and the rate of degradation as demonstrated by the slope of heat flow curves (Fig. 3).

The susceptibility of tyrosine and tryptophan to oxidative degradation was observed to be much lower than in the case of sulphur containing amino acids (cysteine, methionine) (Fig. 4). Heat flow curves of tyrosine and tryptophan solutions are almost parallel with heat flow values greater than zero, indicating oxidation reactions. Although the mass ratio between amino acid and oxidant was adjusted to be approximately the same for all compounds tested the lower tyrosine and tryptophan concentrations in sample solutions (because of poor solubility) is probably the reason that the heat flow signals are less pronounced. The relative stabilities are also in accordance with HPLC results (100, 97, 10.3 and 5.1% degradation in the case of cysteine, methionine, tyrosine and tryptophan, respectively).

A shorter than usual (5 min) equilibration time before data collecting was selected when performing microcalorimetric analyses in order not to lose information at the beginning of an experiment because of the relatively fast reactions in the presence of the oxidant. The advantage of this approach is seen in Fig. 1, where the heat flow signal of ascorbic acid in 0.3% H_2O_2 solution is much greater at the beginning, falling rapidly below the signal of the 0.03% H_2O_2 solution. This could easily be overlooked, leading to misinterpretation of microcalorimetric responses if data were not collected until 0.5 h.

In oxidation studies extra attention must be paid when using metal vessels, because of the ability of metals to catalyze such reactions. We noticed in some cases (e.g. ascorbic acid, tyrosine) differences between degradation of compounds stored in a vessel and in a flask measured at same conditions, suggesting that glass cells should be used in such cases.

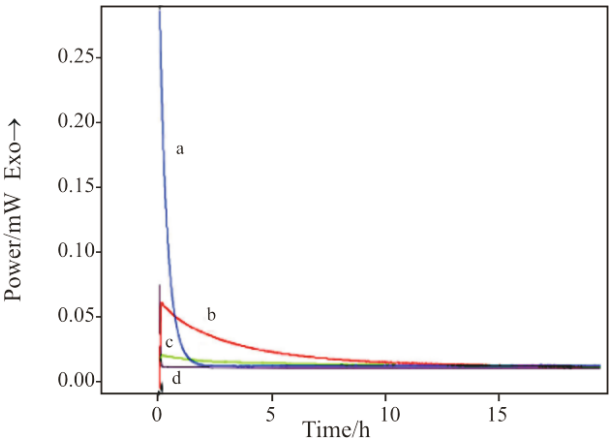

Fig. 2 Power–time curves for the oxidation of methionine in a – 0.3% H_2O_2, b – 0.03% H_2O_2, c – 0.003% H_2O_2, d – water solution

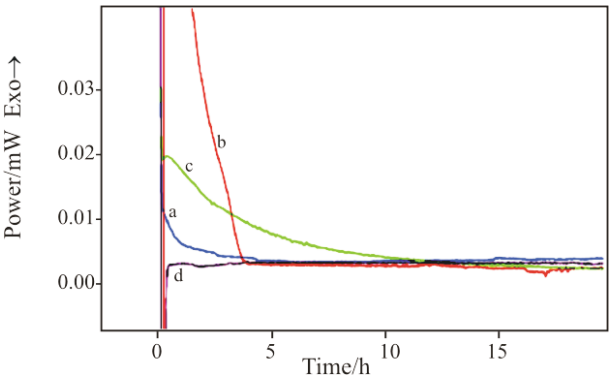

Fig. 3 Power–time curves for the oxidation of cysteine in a – 0.3% H_2O_2, b – 0.03% H_2O_2, c – 0.003% H_2O_2, d – water solution

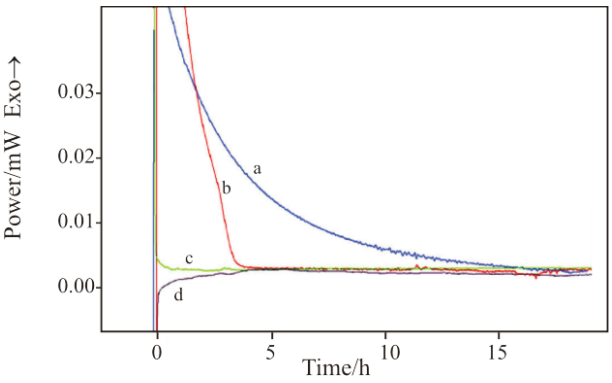

Fig. 4 Power–time curves for the oxidation of different amino acids in hydrogen peroxide solutions. Heat flow outputs are for a – methionine, b – cysteine, c – tyrosine, d – tryptophan

Chromatographic analyses for all tested compounds were performed by the use of a stability indicating HPLC methods, based on a special Synergy Hydro column. This stationary C18 bonded phase endcapped with a unique polar group and with a high

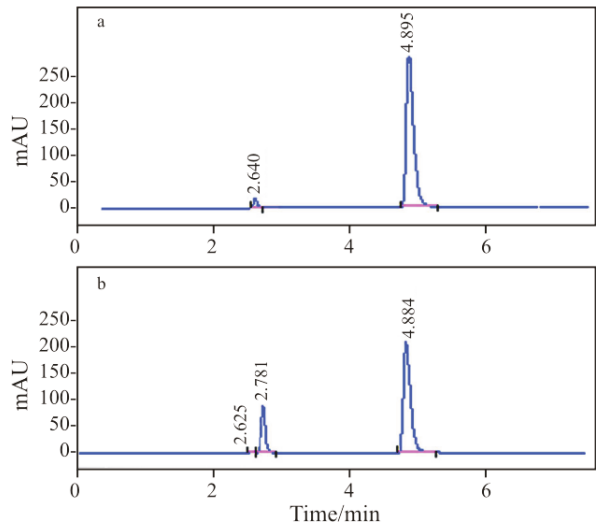

Fig. 5 The chromatograms of methionine (t_R=4.9 min) in 0.003% H_2O_2 solution obtained after a – t=0 and b – t=20 h

4 μm silica surface area combined with a dense bonded phase coverage claimed suitable for analysis of very polar compounds [20], proved also in our case to be very efficient in separating amino acids and their degradation products. Typical chromatograms of methionine in H_2O_2 solution obtained at the beginning and after 20 h are shown in Fig. 5, indicating the good resolution between methionine, hydrogen peroxide and the main degradation product methionine sulfoxide appearing after 20 h.

Conclusions

Based on our experiments we can conclude that isothermal microcalorimetry can be successfully applied as a fast and simple method for prediction of oxidative degradation in the presence of hydrogen peroxide. We proved that, for the compounds studied, isothermal microcalorimetry provides a good index of stability, which is also in accordance with quantitative measurements performed by HPLC showing the decreasing susceptibility to oxidation in the order from sulphur containing amino acids (cysteine, methionine), to ascorbic acid and finally to aromatic amino acids (tyrosine, tryptophan).

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