ELSEVIER

Journal of Chromatography B, 695 (1997) 427–433

JOURNAL OF CHROMATOGRAPHY B

Short communication

Different efficacy of iodoacetic acid and N-ethylmaleimide in high-performance liquid chromatographic measurement of liver glutathione

Gregorio Santori*, Cinzia Domenicotti, Antonella Bellocchio, Maria A. Pronzato, Umberto M. Marinari, Damiano Cottalasso

Institute of General Pathology, University of Genoa, Via L.B. Alberti 2, 16132 Genoa, Italy

Received 14 November 1996; revised 21 February 1997; accepted 27 February 1997

Abstract

The widely used high-performance liquid chromatography (HPLC) procedure to determine glutathione in biological samples utilizing iodoacetic acid as thiol quenching agent and 1-fluoro-2,4-dinitrobenzene for derivatization has been modified regarding tissue sample processing and storage of the working solutions. The modified procedure compared with the original method reduces artifactual oxidation in rat liver glutathione measurement (1.47±0.8% vs. 2.84±0.69%, respectively). In both HPLC procedures, an increase in artifactual oxidation was found in both standard glutathione solutions and hepatic samples when N-ethylmaleimide instead of iodoacetic acid was used for thiol trapping. © 1997 Elsevier Science B.V.

Keywords: Iodoacetic acid; N-Ethylmaleimide; Glutathione

1. Introduction

Glutathione (GSH, L-γ-glutamyl-L-cysteinyl-glycine), an essential thiol ubiquitous in mammalian cells, plays a crucial role in cell protection against oxidative stress [1,2]. Considering that an accurate measurement of both GSH and oxidized glutathione (GSSG) is critical for a reliable GSH/GSSG ratio, it is fundamental to reduce artifactual GSH oxidation during assay procedure. One of the most widely used high-performance liquid chromatography (HPLC) methods to determine both GSH and GSSG in biological samples has been proposed by Reed et al. [3] and subsequently improved by Fariss and Reed [4], and it employs iodoacetic acid (IAA) as a thiol quenching agent. Recently, Asensi et al. have reported that current HPLC methods to quantitate GSH and GSSG should be considered unsuitable because of excessive artifactual oxidation [5]. Therefore, Asensi et al. [5] have developed a modified version of the HPLC procedure of Fariss and Reed that allows only GSSG determination in biological samples using N-ethylmaleimide (NEM) as a thiol quenching agent.

In this work, following the HPLC method of Fariss and Reed, we found more artifactual oxidation in rat liver samples using NEM rather than IAA for thiol trapping (4.96±0.58% vs. 2.84±0.69%, respectively). A slightly modified version of this HPLC method that further reduces the artifactual oxidation

*Corresponding author.

PII S0378-4347(97)00159-X

in both standard GSH solutions and rat liver samples using IAA is also described.

2. Experimental

2.1. Reagents

Methanol, sodium acetate, glacial acetic acid and $KHCO_3$ were of HPLC grade from BDH (Poole, UK). Perchloric acid (PCA) and KOH were from Merck (Darmstadt, Germany). Bathophenanthroline disulfonic acid (BPDS), γ-glutamylglutamate, IAA, NEM, 1-fluoro-2,4-dinitrobenzene, *m*-cresol, GSH and GSSG were from Sigma (St. Louis, MO, USA). Only metal-free H_2O (Milli-Q Reagent Water System, Millipore, Marlborough, MA, USA) was used.

2.2. Standard GSH solutions

GSH was prepared either in 10% PCA with 1 m*M* BPDS or in 10% PCA with 1 m*M* BPDS and 40 m*M* NEM at a concentration of 1084 nmol/ml. The amount of GSSG in PCA solutions containing GSH was calculated from the corresponding PCA solutions containing GSSG to a final concentration of 1084 nmol/ml. The percentage of GSH oxidation in standard GSH solutions was calculated as:

$$\% \text{ oxidation} = A \times 2 \times 100 \times B^{-1}$$

where A = GSSG amount in PCA containing GSH, and B = final concentration of GSH. The factor 2 was used to convert GSSG to GSH equivalents as in Asensi et al. [5]. Standard GSH solutions were derivatized and analyzed by HPLC as described below.

2.3. Liver processing

Livers were excised from male Wistar rats of 200±25 g. Rats were kept under controlled temperature (20±3°C), humidity (60%) and a 12 h light/dark cycle (light on 8.00 a.m.); free access to food and water was allowed; food but not water was denied for 16 h before sacrifice.

Hepatic samples (1 g) were homogenized (1:10 w/v) with: (i) 10% PCA containing 1 m*M* BPDS or 10% PCA containing 1 m*M* BPDS and exogenous GSH as internal standard; (ii) 10% PCA containing 1 m*M* BPDS and 40 m*M* NEM or 10% PCA containing 1 m*M* BPDS, 40 m*M* NEM and exogenous GSH as internal standard. Liver samples homogenized in 10% PCA were promptly centrifuged at 15 000 *g* and 4°C for 3 min. Alternatively, following the original method of Fariss and Reed [4], the acid extract of powdered tissue was sonicated, frozen and thawed, and finally centrifuged. After centrifugation, 0.5 ml of the resulting acid extract was added to 50 μl of 15 m*M* γ-glutamylglutamate (γ-Glu-Glu) in 0.3% PCA [4]. Oxidative artifact was calculated as described by Asensi et al. [5].

2.4. Derivatization

To acid extract from standard GSH solutions and hepatic samples treated with 10% PCA–1 m*M* BPDS, 50 μl of 100 m*M* IAA in a 0.2 m*M* *m*-cresol purple solution was immediately added. Conversely, to acid extract from samples treated with 10% PCA–1 m*M* BPDS–40 m*M* NEM, IAA was not added. Then, all acid solutions were brought to pH 8.0 [6] by the addition of 2 *M* KOH–2.4 *M* $KHCO_3$ and incubated in the dark at room temperature for 10 min [4]. Finally, 1 ml of 1% 1-fluoro-2,4-dinitrobenzene (Sanger's reagent) dissolved in ethanol was added. The reaction mixture was capped, mixed and stored in darkness at 4°C. HPLC analysis was performed within 2 days (Table 1).

2.5. HPLC assay

Standard GSH solutions and hepatic samples were assayed by HPLC as described by Fariss and Reed [4]. A Kontron Instruments (Milan, Italy) 322 HPLC system equipped with a Spherisorb (Phase Separations, Clwyd, UK) NH_2 column (5 μm particle size, 250×4.6 mm I.D.) and a UV absorbance detector (365 nm) was used. A Spherisorb (Phase Separations) NH_2 precolumn (20×4.6 mm I.D.) was placed before the analytical column and replaced when the back-pressure of the HPLC system exceeded 17237.5 kPa. The flow-rate was 1.0 ml/min. Following the 100 μl injection of the derivatized sample, the mobile phase was maintained at 80% A (80% methanol in metal-free H_2O) and 20% B (0.5 *M* sodium acetate in 64% methanol) for 5 min

Table 1
Modifications of the HPLC method of Fariss and Reed [4] for reducing oxidative artifact in the treatment of standard GSH solutions and hepatic samples

	Fariss and Reed	Fariss and Reed modified
10% PCA-1 m*M* BPDS solution	–	Made fresh daily and maintained at 4°C
Standard GSH solution[a]	–	Made fresh daily in PCA–BPDS and maintained at 4°C
Tissue sample	Powdered, sonicated, frozen and thawed, and finally centrifuged	Homogenized (1/10 w/v)[b] in PCA–BPDS and promptly centrifuged
15 m*M* γ-Glu-Glu	Stable at room temperature for several days	Made fresh daily and maintained at 4°C
100 m*M* IAA	Maintains alkylating activity for at least 7 days at 4°C	Used within 3 days; stored at 4°C
HPLC analysis	Within 6 weeks	Within 2 days

[a] It is of great importance to determine the standard curve under the same conditions of the tissue sample, in particular in the presence of the protein precipitant [1]. Under our conditions, 10% PCA did not cause artifactual oxidation in standard GSH solutions.
[b] Homogenization at pH≥2 is undesiderable because of the likelihood of thiol–disulfide interchange and thiol oxidation [1].

followed by a 10-min linear gradient to 1% A and 99% B. The mobile phase was maintained at 99% B until GSSG eluted.

2.6. Statistical analysis

Results are expressed as mean±S.D. for the number of experiments ($n=5$). The statistical significance between sets of experimental data was assessed by Student–Newman–Keuls multiple comparisons test. Statistical analysis was performed using the software program GraphPad InStat (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. GSH oxidation during treatment of standard GSH solutions with IAA

GSH was prepared in 10% PCA with 1 m*M* BPDS at a final concentration of 1084 nmol/ml. Standard GSH solutions were treated with 100 m*M* IAA in 0.2 m*M* *m*-cresol, derivatized and analyzed by HPLC following either the method of Fariss and Reed [4] or a slightly modified version of this same method (Table 1). Following the method of Fariss and Reed [4], GSSG level in PCA solutions containing GSH was 8.53±1.12 nmol/ml, and the percentage of GSH oxidation in the range of 1.56±0.25% (Table 2 IA). When standard GSH solutions were treated according to our modifications of the original procedure, the GSSG amount was found to be 5.55±0.75 nmol/ml and oxidative artifact in the range of 1.02±0.13% (Table 2 IIA).

A typical chromatogram of a standard GSH solution trapped with IAA shows one GSH peak (Fig.

Table 2
Glutatione oxidation in standard GSH solutions

	GSSG level (nmol/ml)	
	I. Fariss and Reed	II. Fariss and Reed modified
A. IAA	8.53±1.12	5.55±0.75
% oxidation	1.56±0.25[a]	1.02±0.13[a]
B. NEM	51.99±9.00	29.02±5.79
% oxidation	9.56±1.65	5.33±1.06[b]

Note: We measured GSSG level in standard GSH solutions (final concentration 1084 nmol/ml) following the original method of Fariss and Reed [4] (I), and the same method modified as described in Table 1 (II), using IAA (A) or NEM (B) as thiol trapping agent. The percentage of GSH oxidation was calculated as described in Section 2. Values are means±S.D. ($n=5$). Statistical significance between the percentages of oxidation was calculated using Student–Newman–Keuls multiple comparisons test.
[a] $P<0.001$ for AI vs. BI, and AII vs. BII.
[b] $P<0.001$ for BII vs. BI.

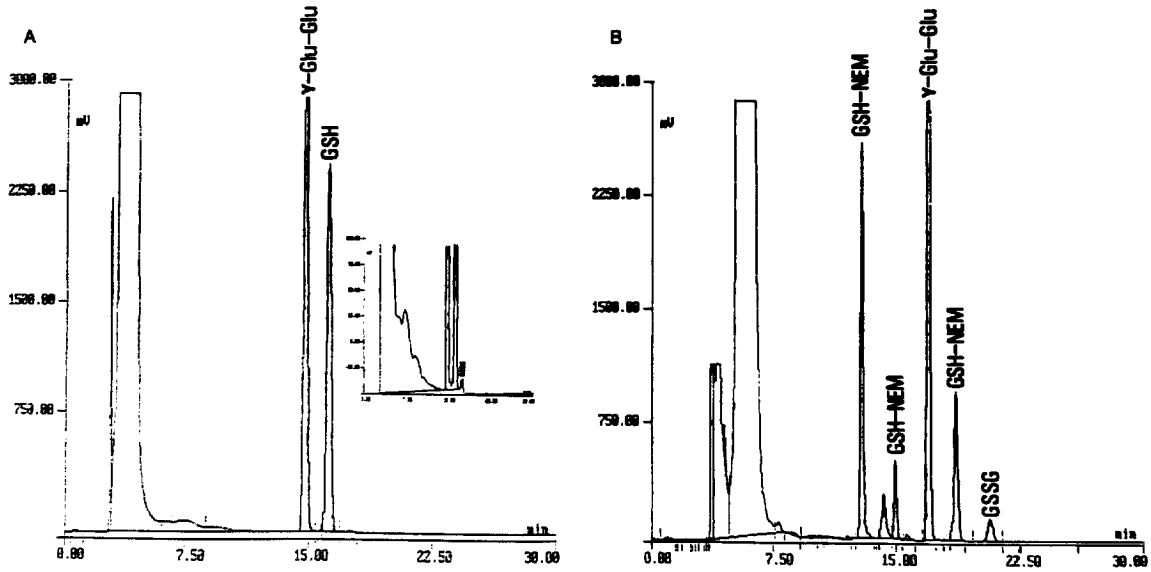

Fig. 1. (A) Chromatogram of a standard GSH solution (final concentration 1084 nmol/ml) treated with IAA, derivatized and analyzed by HPLC as described by Fariss and Reed [4] with modifications described in Section 2. In the inset, the mV scale (y-axis) was reduced 30-fold and GSSG peak enhanced (5.30 nmol GSSG ml^{-1}). (B) Chromatogram of the same standard GSH solution derivatized and analyzed by HPLC as (A) but using NEM as a thiol trapping agent (32.15 nmol GSSG ml^{-1}).

1A), and the GSSG peak becomes visible only by 30-fold reduction of the mV scale (Fig. 1A, inset).

3.2. GSH oxidation during treatment of standard GSH solutions with NEM

GSH was prepared in 10% PCA with 1 m*M* BPDS and 40 m*M* NEM at a final concentration of 1084 nmol/ml. Standard GSH solutions were derivatized and analyzed by HPLC following either the method of Fariss and Reed [4] or a slightly modified version of this same method (Table 1). Following the method of Fariss and Reed [4], the amount of GSSG in PCA–NEM solutions containing GSH was 51.99±9.00 nmol/ml, and the percentage of GSH oxidation in the range of 9.56±1.65% (Table 2 IB). According to our modifications of the original method, the GSSG level was found to be in the range of 29.02±5.79 nmol/ml and the oxidative artifact in the range of 5.33±1.06% (Table 2 IIB).

Chromatograms of standard GSH solutions trapped with NEM show both the GSH–NEM adduct, decomposed into two or three peaks, and the GSSG peak without reducing the mV scale (Fig. 1B).

3.3. GSH oxidation during treatment of hepatic samples with IAA

Hepatic samples were treated following either the method of Fariss and Reed [4] or a modified version of this same method (Table 1). In both procedures, 100 m*M* IAA in *m*-cresol was added to a portion of the resulting acid extract. Derivatization and HPLC analysis were performed as described in Section 2. Following the original method [4], hepatic samples treated with IAA contained 426.18±33.12 nmol GSSG g^{-1} and after addition of 1084 nmol GSH g^{-1} we found 450±38 nmol GSSG g^{-1}, which is equivalent to GSH oxidation of 2.84±0.69% (Table 3 IA). When hepatic samples were processed according to our modifications of the original procedure, we found 204.60±25.18 nmol GSSG g^{-1}, and after addition of 1084 nmol GSH g^{-1} we found 218.15±20 nmol GSSG g^{-1}, which is equivalent to GSH oxidation of 1.47±0.8% (Table 3 IIA).

Chromatograms of hepatic samples treated with IAA allow HPLC measurement of both GSH and GSSG, with the occurrence of only a single GSH peak (Fig. 2A).

Table 3
Glutathione oxidation in hepatic samples

	GSSG level (nmol/g)			
	I. Fariss and Reed		II. Fariss and Reed modified	
	A. IAA	B. NEM	A. IAA	B. NEM
− GSH	426.18±33.12	481.17±35.7	204.60±25.18	396.35±28.3
+ GSH	450±38	560.12±40	218.15±20	450±23
% oxidation	2.84±0.69[a]	4.96±0.58	1.47±0.8[a,b]	4.6±0.78

Note: We measured GSSG level in hepatic samples following the original method of Fariss and Reed [4] (I), and the same method modified as described in Table 1 (II), using IAA (A) or NEM (B) as thiol trapping agent. The percentage of GSH oxidation after addition of 1084 nmol GSH g^{-1} was calculated as described by Asensi et al. [5]. Values are means±S.D. ($n=5$). Statistical significance between the percentages of oxidation was calculated using Student–Newman–Keuls multiple comparisons test.
[a] $P<0.001$ for A I vs. B I, and A II vs. B II.
[b] $P<0.01$ for A II vs. A I.

3.4. GSH oxidation during treatment of hepatic samples with NEM

Hepatic samples were treated following either the method of Fariss and Reed [4] or a modified version of this same method (Table 1), using NEM instead of IAA for thiol trapping as described in Section 2. Following the method of Fariss and Reed [4], we found 481.17±35.7 nmol GSSG g^{-1}, and after addition of 1084 nmol GSH g^{-1} we found 560.12±40 nmol GSSG g^{-1}, which is equivalent to GSH oxidation of 4.96±0.58% (Table 3 IB). By introducing minor modifications to the procedure, we found 396.35±28.3 nmol GSSG g^{-1} and after addition of 1084 nmol GSH g^{-1} we found 450±23 nmol GSSG g^{-1}, which is equivalent to GSH oxidation of 4.6±0.78% (Table 3 IIB).

Chromatograms of hepatic samples treated with

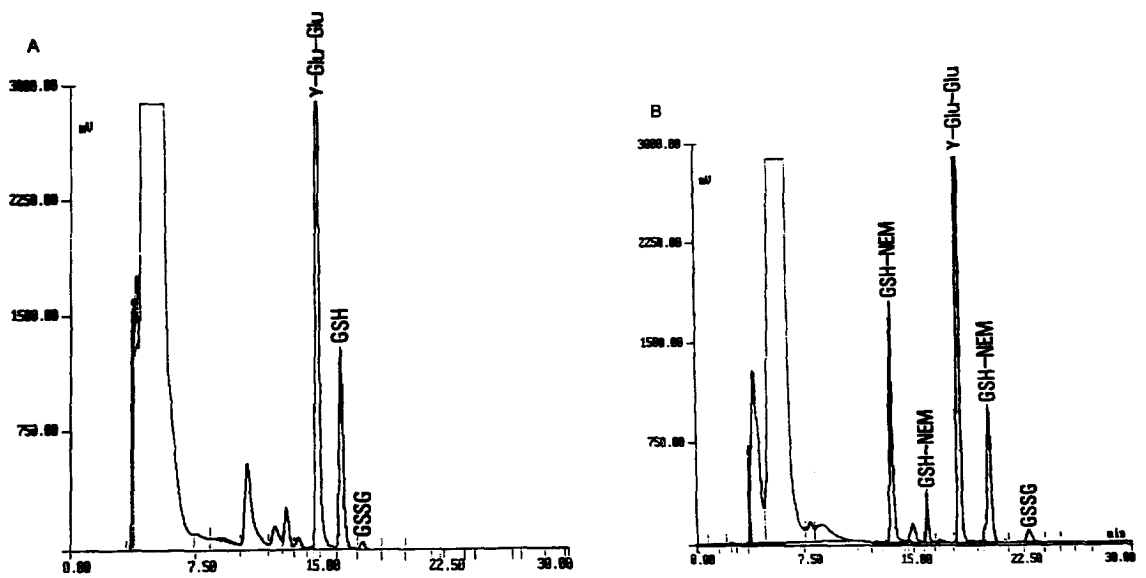

Fig. 2. (A) Chromatogram of hepatic sample derivatized and analyzed by HPLC as described by Fariss and Reed [4] with modifications described in Section 2 and using IAA as a thiol trapping agent (4915.85 nmol GSH g^{-1}, 185.37 nmol GSSG g^{-1}). (B) Chromatogram of hepatic sample derivatized and analyzed by HPLC as (A) but using NEM as a thiol trapping agent (384.26 nmol GSSG g^{-1}).

NEM show the GSH–NEM adduct decomposed into two or three peaks, and a single GSSG peak (Fig. 2B).

Both the replacement of IAA with NEM in the step where IAA was originally used and the replacement of NEM with IAA in the liver homogenization resulted in much higher GSH oxidation (data not shown).

4. Discussion

The widely used HPLC method of Fariss and Reed [4] allows the determination of both GSH and GSSG in biological samples utilizing IAA for thiol trapping and 1-fluoro-2,4-dinitrobenzene for derivatization. Recently, Asensi et al. reported that this HPLC method should not be considered suitable for GSSG determination in blood or tissue like liver or kidney owing to excessive artifactual GSH oxidation [5]. Thus, Asensi et al. developed a modified version of this HPLC assay that allows only GSSG determination in biological samples using NEM instead of IAA for thiol quenching [5]. In contrast to the HPLC method of Mansoor et al. [7], where GSH and GSSG may be assayed in separate samples utilizing monobromobimane for derivatization, following the method of Asensi et al. [5] GSH cannot be measured by HPLC because the GSH–NEM adduct is decomposed into three peaks, resulting in a GSH/GSSG ratio calculated without utilizing the same analytical procedure.

In this work, we evaluated the effectiveness of IAA and NEM as thiol quenching agents on both standard GSH solutions and rat liver samples. Sample processing, derivatization and HPLC analysis were carried out following the original method of Fariss and Reed [4] and the same method with modifications described in Section 2 (Table 1). When standard GSH solutions were treated with IAA and analyzed by HPLC following the method of Fariss and Reed, we found more GSH oxidation (Table 2 IA) than following the same method with our modifications (Table 2 IIA). In both procedures, when standard GSH solutions were trapped using NEM instead of IAA, an increase in oxidative artifact was noted (Table 2 B). GSH oxidation in hepatic samples treated with IAA and assayed by the method of Fariss and Reed was in agreement with the values obtained by Asensi et al. following the same method [5] (Table 3 IA). By applying a slightly modified version of the procedure of Fariss and Reed (Table 1), we found a further decrease in GSH oxidation (Table 3 IIA). In both procedures, when hepatic samples were treated with NEM instead of IAA, a significant increase in oxidative artifact was calculated (Table 3 B).

It is likely that tissue processing, as well as storage of working solutions and derivatized samples, can influence the amount of oxidative artifact. In this way, effectiveness of the thiol quenching agent used certainly plays a critical role. Quenching agents differ widely with respect to the rate at which they react with thiols [8]. Thiols may be efficiently trapped in a stable form by adding 100 m*M* IAA [6], and alkylation of thiols with this concentration of IAA occurs with a half-time of about 1 s at pH 8 [9]. However, at a concentration of 100 m*M* NEM, the half-time for the alkylation of thiols at pH 8 would be about 0.7 ms [8], making the use of this thiol quenching agent especially suitable for blood samples [5,7]. NEM is generally preferred because of its rapid reaction rate (completion within 1 min) in contrast to IAA (5 to 15 min) [5,10–12], although concentrations lower than 100 m*M* are used [5]. Conversely, in some procedures the use of NEM has been associated with erroneously high values of GSSG [13]. Interestingly, both the reversibility of NEM alkylation of GSH [14] and the hydrolysis of NEM [15] can occur in a basic medium [5]. Considering that NEM alkylation is potentially not irreversible [9], limited effectiveness of NEM observed in our conditions could be explained in view of unstable alkylation.

Using IAA instead of NEM and introducing minor modifications to the method of Fariss and Reed, a reliable HPLC assay of both GSH and GSSG in hepatic samples is achieved.

Acknowledgments

We wish to thank Mr. Giuseppe Catalano and Mr. Giuseppe Maloberti for technical assistance. This study was supported by grants from CNR (Project ACRO) and MURST 40% and 60%.

References

[1] A. Meister, M.E. Anderson, Annu. Rev. Biochem. 52 (1983) 711.
[2] A. Meister, in D. Dolphin, R. Poulson and O. Avramovic (Editors), Glutathione: Chemical, Biochemical and Medical Aspects, Wiley, New York, 1989, p. 367.
[3] D.J. Reed, J.R. Babson, P.W. Beatty, A.E. Brodie, W.W. Ellis, D.W. Potter, Anal. Biochem. 106 (1980) 55.
[4] M.W. Fariss, D.J. Reed, Methods Enzymol. 143 (1987) 101.
[5] M. Asensi, J. Sastre, F.V. Pallardó, J.G. de la Asunción, J.M. Estrela, J. Viña, Anal. Biochem. 217 (1994) 323.
[6] T.E. Creighton, Methods Enzymol. 131 (1986) 83.
[7] M.A. Mansoor, A.M. Svardal, P.M. Ueland, Anal. Biochem. 200 (1992) 218.
[8] H.F. Gilbert, Methods Enzymol. 251 (1995) 8.
[9] J.S. Weissman, P.S. Kim, Science 253 (1991) 1386.
[10] F.A.M. Redegeld, A.S. Koster and W.P. Van Bennekom, in J. Viña (Editor), Glutathione: Metabolism and Physiological Functions, CRC Press, Boca Raton, FL, 1990, p. 11.
[11] T.P.M. Akerboom, H. Sies, Methods Enzymol. 77 (1981) 373.
[12] D. Gallemann, P. Eyer, Anal. Biochem. 191 (1990) 347.
[13] E. Beutler, C. West, Anal. Biochem. 81 (1977) 458.
[14] E. Beutler, S.K. Srivastava, C. West, Biochem. Biophys. Res. Commun. 38 (1970) 341.
[15] P. Sacchetta, D. Di Cola, G. Federici, Anal. Biochem. 154 (1986) 205.