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High-performance liquid chromatographic assay of hydroxyl free radical using salicylic acid hydroxylation during in vitro experiments involving thiols

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Abstract

A HPLC method was developed to monitor the production of hydroxyl free radical (°OH) produced during in vitro experiments: (i) a chemical reaction involving EDTA chelated ferric ion and various exogenous and endogenous thiols [glutathione (GSH) and its metabolites], and (ii) an enzymatic reaction corresponding to the breakdown of GSH catalyzed by γ-glutamyltransferase (GGT). The method relies upon the use of a selective trapping reagent of °OH: salicylic acid (SA). The three resulting dihydroxylated products, i.e., 2,3-dihydroxybenzoic acid (DHB), 2,5-DHB and catechol, were measured in an ion-pairing reversed-phase HPLC system coupled with amperometric detection; the sum of the three concentrations was used to quantify the production of °OH during in vitro experiments. Resulting data demonstrate that °OH is produced during Fenton-like reactions involving thiols and GSH catabolism via GGT.

Keywords: Hydroxyl free radical; Salicylic acid; Thiols

1. Introduction

Thiol-containing compounds play an important role in protecting biological systems against oxidative injury; they interact either directly with oxidants, especially reactive oxygen species (ROS) as strong reducing chemical compounds, or by enzymatic mechanism, e.g., glutathione (γ-glutamylcysteinylglycine; GSH). GSH is the main intracellular low-molecular-mass thiol; it is implied in numerous antioxidant mechanisms, and is also the co-substrate of enzymes involved in detoxification processes, such as glutathione-S-transferases and glutathione peroxidases [1].

However, in the presence of molecular oxygen and iron or copper ions [2], a number of antioxidants including thiols paradoxically generate ROS, leading to cellular signaling [3] or to free radical damage of cell components [4,5]. Intracellular GSH is maintained at a constant level, by regulation of its de novo synthesis, as described by Meister and Anderson [6]. γ-Glutamyltransferase (GGT; E.C. 2.3.2.2),

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a plasma membrane enzyme, is able to initiate extracellular GSH breakdown and the resulting products are further transferred to the cells for GSH synthesis. However, the simultaneous production of ROS during the GGT reaction has been recently reported [5,7].

The study of anti- and pro-oxidant effects occurring during thiol oxidation and GSH metabolism requires methodological tools to assess the production of ROS. Usual methodologies devoted to ROS measurement are electronic spin resonance (ESR) with spin trapping reagents [8], chimioluminescence [5], fluorogenic probes, mainly 2′,7′-dichlorofluorescin and dihydrorhodamine 123 (DHR 123) [9], associated with flow cytometry [10] or capillary electrophoresis [11], and hydroxylation of aromatic compounds [12]. These different methods exhibit various specificity with regard to the different ROS. For example, the fluorogenic reagent DHR 123 is reported to be oxidized to the fluorescent rhodamine 123 by a wide variety of ROS: superoxide anion, hydrogen peroxide in presence of peroxidase, hydroxyl radical (°OH) [11] and peroxinitrite [13].

Aromatic hydroxylation is one of the most specific and sensitive methods for °OH measurement; it relies upon the ability of this radical which is very toxic, has a short half-life and therefore is present at extremely low concentrations in biological systems, to attack the phenyl ring of aromatic molecules and produce hydroxylated compounds that can be directly measured by high-performance liquid chromatography (HPLC) coupled with electrochemical detection (ED) [12,14–18] or more recently by capillary electrophoresis [19]. Phenylalanine, benzoic acid and salicylic acid (SA) or its esterified form aspirin have been reported for this purpose but SA has been mainly used because of (i) its high reaction rate ($5 \cdot 10^9$ M^{-1} s^{-1}), (ii) its use at concentrations sufficient to compete with other present scavengers and (iii) the stability of resulting products which facilitates further sample treatment and analytical processes [12]. Thus, measuring °OH by monitoring products resulting from attack of SA by this radical is probably an easier way and a better approach than using 5,5-dimethylpyrroline-*N*-oxide (DMPO) as a spin trapping reagent because the resulting adduct DMPO–°OH exhibits poor stability and no authentic standard of this adduct is available for quantitation by HPLC–ED [8].

Whereas ESR has been used widely to monitor the production of °OH by thiols, the use of hydroxylation of SA was reported only once [20] and the corresponding method used was colorimetry. The present work deals with the development of a HPLC technique for °OH measurement via the hydroxylation of SA, which is claimed to be a highly selective °OH trapping reagent [12]. In contrast to previously reported techniques, the HPLC system was devoted to the separation and quantification of the three resulting products, 2,3-dihydrobenzoic acid (DHB), 2,5-DHB and catechol (Fig. 1) with full selectivity with regard to the thiols involved during

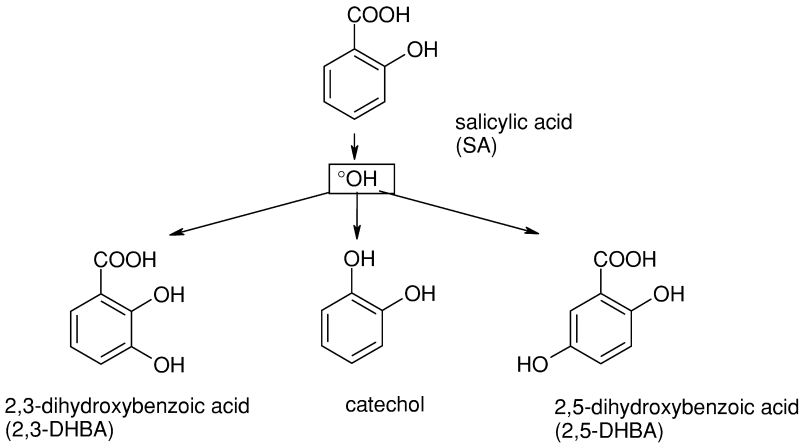

Fig. 1. Reaction scheme of salicylic acid hydroxylation by hydroxyl radicals (°OH).

experiments. It consists of an ion-pairing reversed-phase HPLC system coupled with amperometric detection, which was optimized and validated. It was then applied to quantitatively monitor the °OH production during in vitro experiments involving: (i) a chemical reaction in the presence of various endogenous thiols (GSH and its metabolites) and exogenous thiol substances (chemical reagents and drugs), and ferric ion chelated with EDTA, (ii) an enzymatic reaction corresponding to the breakdown of GSH catalyzed by GGT.

2. Experimental

2.1. Chemicals and reagents

All chemicals were of analytical-reagent grade and used without further purification. Thiols, SA, catechol, 2,3-DHB and 2,5-DHB were purchased from Sigma–Aldrich (St. Quentin-Fallavier, France).

2.2. In vitro °OH generating systems

2.2.1. Thiol/Fe^{3+}/EDTA system

The thiol-dependent °OH generating system includes 0.4 m*M* of each thiol tested, 0.15 m*M* Fe^{3+} and 0.165 m*M* EDTA premixed solutions and SA at concentrations ranging from 0.2 to 3 m*M*. All the solutions were realized in phosphate-buffered saline (PBS), pH 7.4 (Euromedex, Mundolsheim, France).

All the reactions were started by adding the thiol solution and incubating at 37°C. Aliquots of 200 μl were withdrawn at various time intervals, added to 50 μl of 1 *M* hydrochloric acid, kept in ice for a maximum of 12 h or at −80°C until HPLC analysis.

2.2.2. GGT/glutathione/Fe^{3+}/EDTA system

Cell pellets corresponding to 10^6 cultured GGT related cDNA transfected cells or control mock transfected cells (murine NIH 3T3 GGT-rel or NIH 3T3 fibroblasts [5,21]) were gently washed in PBS, pH 7.4 then suspended in 1 ml PBS in the presence of GSH (2.5 m*M*), glycylglycine (glygly; 25 m*M*), Fe^{3+} (0.150 m*M*), EDTA (0.165 m*M*) and SA (2 m*M*). During a 3-h incubation period at 37°C, 200-μl aliquots were withdrawn at various time intervals and mixed with 50 μl of 1 *M* hydrochloric acid. The resulting mixture was centrifuged at 5000 *g* for 5 min at 4°C and the supernatant was kept in ice for a maximum of 12 h or at −80°C before analysis by HPLC–ED.

2.3. HPLC measurement of hydroxylated products of salicylic acid

The HPLC system included an isocratic pump (Model PU 980, Jasco, Japan), a valve injector (Model Rheodyne 7725i) with a 20-μl loop, a Spherisorb ODS-2 (5 μm) 125×4 mm I.D. column (Macherey Nagel, Duren, Germany) and a LiChrospher RP18 end capped (5 μm) 4×4 mm I.D. guard column (Merck, Darmstadt, Germany), an amperometric detector including a column oven (Model EC 2000, Antek, The Netherlands) and an integrator (Model D-2000, Hitachi–Merck, Germany).

The mobile phase consisted of 30 m*M* citric acid, 5 m*M* tetrabutylammonium bromide and 2.5 m*M* EDTA aqueous solution adjusted to pH 4.5 with 10 *M* NaOH, methanol and tetrahydrofuran (94:4:2, v/v/v). Elution was performed at a flow-rate of 1.2 ml min^{-1} and at a column temperature of 35°C. Amperometric detection was operated using a glassy carbon electrode at an applied potential of +0.45 V vs. H_2/H^+ (Pd electrode). Equilibration time before starting injections, was within 2 h and the mobile phase was recycled for 1 week even during sample analysis without any important baseline drift and noise level increase.

Stock solutions of standards were prepared in methanol at a concentration of 6.5 μ*M* for 2,3-DHB, 2,5-DHB and 9.1 μ*M* for catechol, and stored at +4°C for 1 month. Further dilutions in the mobile phase were realized daily to obtain calibration curves ranging from 0.065 to 1.14 μ*M* for 2,3-DHB and 2,5-DHB and from 0.045 to 0.79 μ*M* for catechol.

Chromatographic parameters were calculated according to the European Pharmacopoeia. The hold-up time t_0 was determined by injecting a nitrite solution into the HPLC system.

Because the highly reactive °OH readily interacts with chemicals other than SA acting as ROS scavengers, the sum of the molar concentrations of 2,3-DHB, 2,5-DHB and catechol reflected the concentration of °OH rather than representing its absolute amount in the incubation medium.

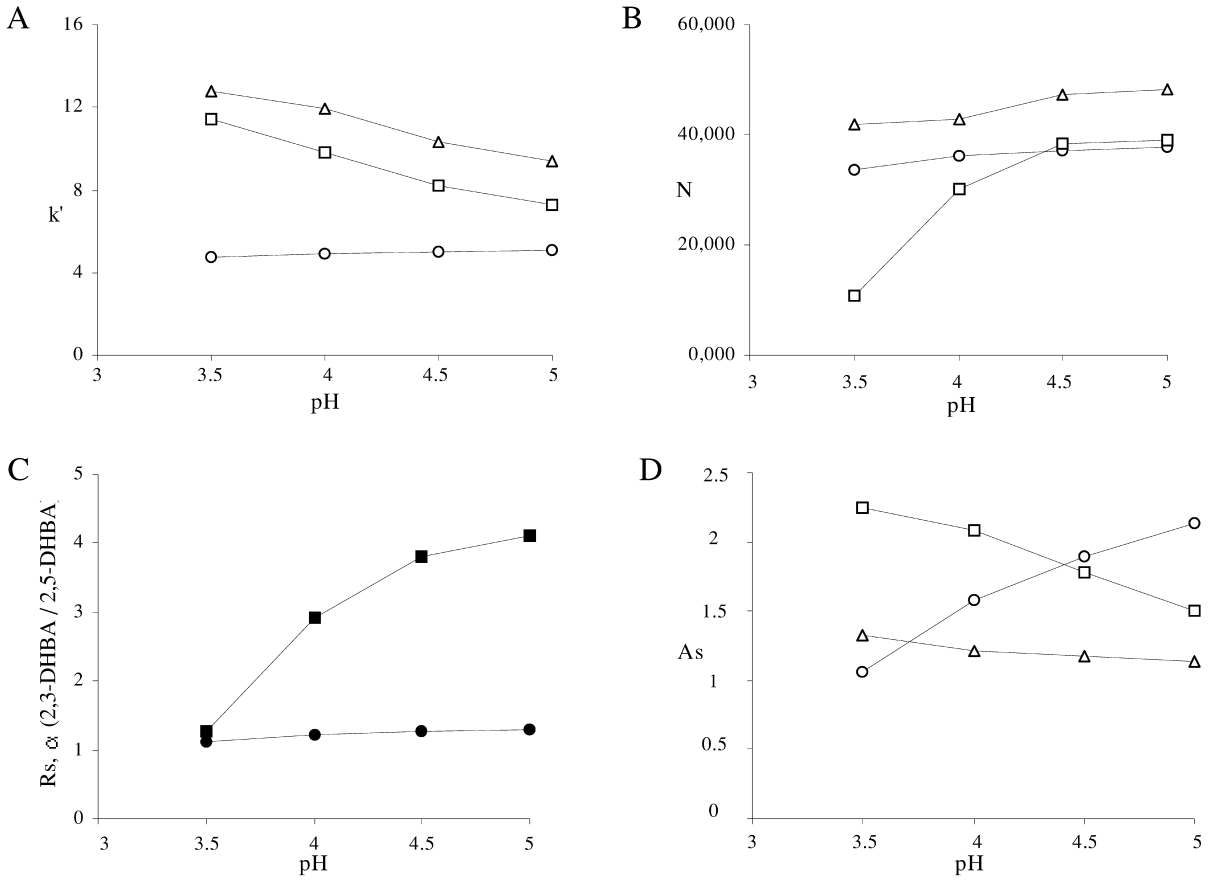

Fig. 2. Influence of the mobile phase pH on chromatographic parameters of analytes: (A) capacity factor (k'), (B) number of theoretical plates per meter (N) and (D) asymmetrical factor (A_s) for catechol (○), 2,3-DHBA (□) and 2,5-DHBA (△); (C) resolution (R_s; ■) and selectivity (α; ●) between 2,3-DHBA and 2,5-DHBA. HPLC operating conditions: column: Spherisorb ODS-2 (5 μm) 125×4 mm I.D.; temperature: 35°C; mobile phase: citrate buffer added with 5 m*M* tetrabutylammonium bromide and 2.5 m*M* EDTA–methanol–tetrahydrofuran (94:4:2, v/v/v); flow-rate: 1.2 ml min^{-1}; amperometric detection: applied potential = +0.45 V vs. H_2/Pd.

3. Results and discussion

3.1. Development of a HPLC system for the measurement of hydroxylated products of salicylic acid

Previously reported studies using hydroxylation of SA as °OH marker during in vivo experiments have assessed that the isomer 2,3-DHB is the only reliable marker for this purpose [12]. As a matter of fact, 2,5-DHB is produced by either phase I metabolism catalyzed by cytochromes P 450 or direct hydroxylation under reaction with °OH, 2,3-DHB only being produced by this latter pathway. The resulting approach is the assay of either 2,3-DHB [14] or the ratio 2,3-DHB/SA [15,16] to monitor in vivo °OH production. Thus, previously reported systems have been developed for this purpose.

During in vitro experiments involving Fenton-like reactions, both isomers, 2,3- and 2,5-DHB, appeared in equal amounts and the sum of their concentrations has been considered to reflect the total °OH amount produced; catechol has been considered to be a minor product of the °OH attack of SA [12]. In preliminary experiments using the thiol/Fe^{3+}/EDTA system, we observed that catechol, 2,3-DHB and 2,5-DHB were produced in proportions of 19–24, 32–41 and 40–46%, respectively; these proportions

were slightly varying according to the thiol tested, but for a given thiol, no significant variation was observed as a function of the reaction time.

Thus, we considered that catechol was not negligible in order to quantify more accurately the °OH production during our experiments. We developed a HPLC system suitable for the separation of the three products of SA hydroxylation by °OH attack. Most of the previously reported HPLC techniques are not able to completely separate these substances within a short time. They rely upon reversed-phase sometimes with addition of a counter ion, sodium octylsulfate [17,18]. Surprisingly, this latter has a negative charge and we preferred to test a quaternary ammonium substance, i.e., tetrabutylammonium bromide (TBABr), which is generally used for the ion-pairing of carboxylic acids in HPLC systems.

The chromatographic conditions were optimized with regard to: (i) the mobile phase composition and (ii) the detection potential.

(i) Addition of TBABr to the mobile phase at a concentration of 5 m*M* has the essential benefit to give baseline resolution between the two isomers of dihydroxysalicylic acid, mainly because of a considerable improvement of 2,3-DHB peak shape. The pH of mobile phase was then tested between 3.5 and 5; this range includes the ionization constants (pK_a) of the 2,3-DHB and 2,5-DHB carboxylic group, thus pH in this region is expected to regulate chromatographic behavior of these analytes. The pH influence on peak parameters of the three analytes, i.e., capacity factors (k'), number of theoretical plates (N), selectivity (α), resolution (R_s) and asymmetrical factor (A_s) values, is given in Fig. 2. First, no variation of k' was observed for catechol because it remains in its molecular form in the tested pH range and a decrease of k' for 2,3-DHB and 2,5-DHB when pH increases was noted (Fig. 2A). An optimum pH value of 4.5 for the mobile phase buffer was selected because it provides the best compromise between short k' (Fig. 2A) and high N values (Fig. 2B): within 40 000 plates m^{-1}. Selectivity (α) between the two isomers (2,3-DHB and 2,5-DHB) was not affected by pH variation but R_s considerably increases when pH raises, and reaches a high value close to 4 at pH 4.5 (Fig. 2C). The retained pH value also affords the best compromise for peak shapes of catechol and 2,3-DHBA (close A_s values) (Fig. 2D).

(ii) Optimization of the detection potential was necessary because the electro-oxidation process of dihydroxylated SA products and the potential of reference electrode, i.e., H_2/Pd, depend of mobile phase pH (Fig. 3). The retained value was $E=+0.45$ V vs. H_2/Pd, which gives the highest cell current level for the three analytes. Furthermore, this low potential value implies a high selectivity, especially vs. SA which is not detected at a potential lower than 0.6 V; this fact allows a high throughput of samples especially when considering SA is used in a large excess in experiments and is late eluted in the present conditions (ca. 30 min). No interference of thiols was noted because they are not detected at this potential on a glassy carbon electrode.

Sample treatment was simple: it consists of an acidification step of the reaction medium and further dilution or centrifugation to eliminate proteins and neither a liquid–liquid extraction step nor use of an internal standard was needed to obtain the required selectivity, precision and accuracy. No degradation of products was observed both in reaction medium, probably because of the presence of EDTA and thiols, and in acidic solution as already reported [16].

Typical chromatograms corresponding to standards and an authentic sample and validation data are shown in Fig. 4 and Table 1, respectively. The

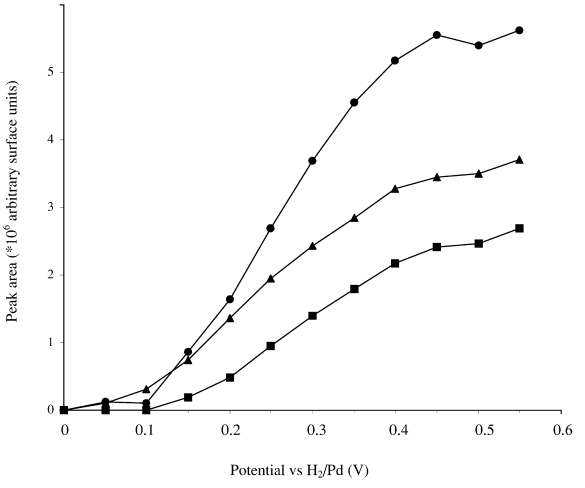

Fig. 3. Hydrodynamic voltamperogram of catechol (●), 2,3-DHBA (■) and 2,5-DHBA (▲) obtained with the amperometric detector equipped with a glassy carbon working electrode; injected amount: 4 ng of each analyte.

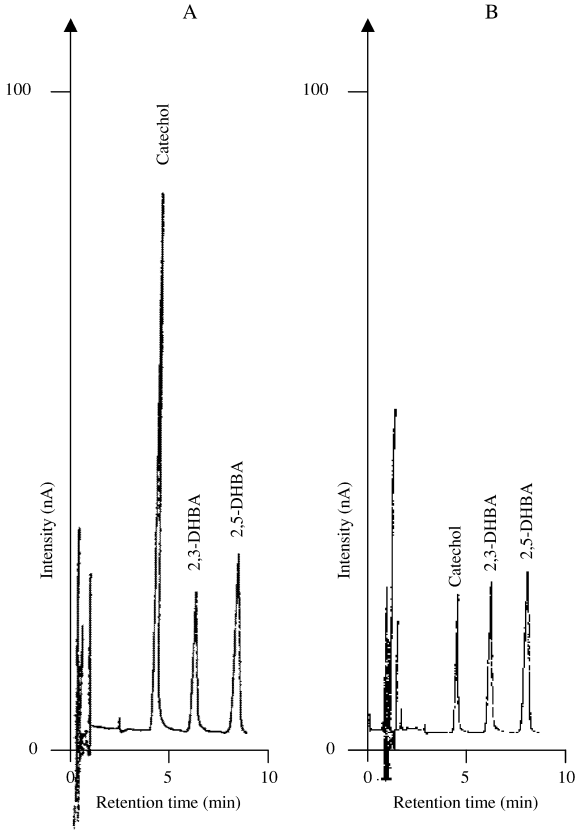

Fig. 4. Typical chromatograms corresponding to (A) standards (catechol: 0.37 μ*M*, 2,3-DHBA and 2,5-DHBA: 0.55 μ*M*) and (B) an authentic sample: incubation medium added with GSH, EDTA chelated Fe^{3+} and glycylglycine in presence of cultured cells expressing GGT.

detection limit (0.013–0.02 μ*M*) obtained with ED was in our hands ca. 100-fold lower than the value found by UV detection.

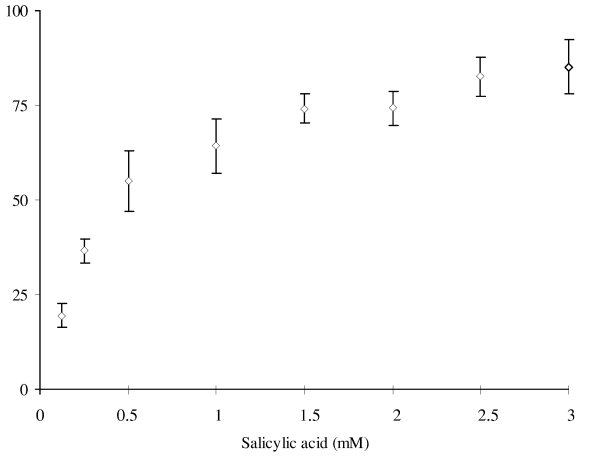

Fig. 5. °OH production monitored using different concentrations of salicylic acid (0.125 to 3 m*M*). °OH concentration corresponds to the sum of catechol, 2,3-DHBA and 2,5-DHBA concentrations measured using HPLC–ED. Cysteine, Fe^{3+} and EDTA concentrations in PBS, pH 7.4 were 0.4, 0.15 and 0.165 m*M*, respectively (incubation temperature: 37°C; reaction time: 60 min). Results from three independent experiments are expressed as means±SD.

3.2. In vitro experiments

Chemical substances with a sulfhydryl group have a high reducing potential (apparent E'° at pH 7.4 in the range −200 to −250 mV). They react with ROS, e.g., superoxide anion ($O_2^{\cdot -}$) with various rate constants to give their corresponding disulfide [22]. Moreover, thiols in presence of ferric ion chelated with EDTA, generate different ROS, and among them probably °OH [2,7] which is one of the highest toxic radical species. Other complexes of biological interest containing Fe(III) or Cu(II), e.g., Fe–ADP, Fe–citrate, ferritine, transferrine and Cu–histidine

Table 1
Main validation parameters obtained for the HPLC assay of hydroxylation products of salicylic acid

Analyte	Linearity range (μ*M*) (six concentration levels)	Regression equation	Correlation coefficient (r^2)	RSD (%)($n=6$)		Detection limit (μ*M*)[a]
				A	B	
Catechol	0.045–0.79	$y=27\,237x-34\,570$	0.997	1.1	4.7	0.013
2,3-DHBA	0.065–1.14	$y=11\,241x-45\,464$	0.999	2.0	6.4	0.02
2,5-DHBA	0.065–1.14	$y=16\,068x-99\,366$	0.998	0.7	5.9	0.02

A: Tested at the highest concentration of the linearity range; B: tested at the lowest concentration of the linearity range.
[a] Measured for a signal-to-noise ratio of 3.

also initiate a ROS production in presence of thiols [5]. The proposed mechanism of thiol-mediated generation of ROS is as follows:

$[pH > 7]$

$RSH \rightarrow RS^-$ (thiolate ion)

$RS^- + Fe^{3+} \rightarrow RS^\circ$ (thiyl radical) $+ Fe^{2+}$

$2RS^\circ \rightarrow RSSR$ (disulfide)

$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\circ -}$ (superoxide anion)

$2O_2^{\circ -} + 2H^+ \rightarrow O_2 + H_2O_2$ (hydrogen peroxide)

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^\circ OH$ (hydroxyl radical)

The aim of the present work is to identify and quantify °OH production rising during reaction of thiols with Fe^{3+}/EDTA chelate.

First, we performed an optimization of SA concentration, which allowed to choose a non-limiting concentration for measurement of °OH in the cysteine/Fe^{3+}/EDTA system (Fig. 5): the plateau of °OH production corresponding to the sum of the three hydroxylated products of SA was reached at 1.5 m*M* and the retained value in further experiments was 2 m*M* of SA.

Various endogenous thiols were tested for their capacity to produce °OH in the presence of chelated iron. All the tested thiols are linked by their metabolism: cysteine (Cys) is synthesized from homocysteine (Hcy) as a precursor and metabolized to other thiol compounds, γ-glutamylcysteine (γ-glucys), glutathione (GSH), cysteamine (Cys-NH_2) and *N*-acetylcysteine (NAC). Cysteinylglycine (Cysgly) and cysteine (Cys) are the breakdown metabolites of GSH via GGT and dipeptidase-catalyzed reactions. We found that Cysgly and Cys exhibited a higher

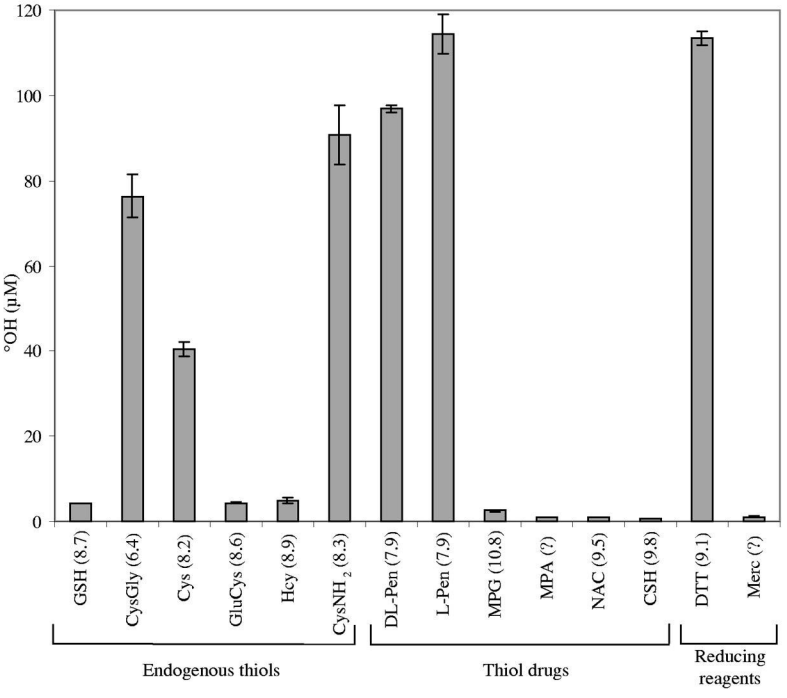

Fig. 6. °OH production using different endogenous and exogenous thiols. Thiol, Fe^{3+} and EDTA concentrations in PBS, pH 7.4 were 0.4, 0.15 and 0.165 m*M*, respectively (incubation temperature: 37°C; reaction time: 60 min). Cys: Cysteine; Cysgly: cysteinylglycine; GSH: glutathione; Hcy: homocysteine; Glucys: γ-glutamylcysteine; $CysNH_2$: cysteamine; D,L-Pen: D,L-penicillamine; L-Pen: L-penicillamine; MPG: mercaptopropionylglycine; MPA: mercaptopropionic acid; CSH: captopril; NAC: *N*-acetylcysteine; DTT: 2,4-dithio-D,L-threitrol; Merc: 2-mercaptoethanol. Values in parentheses are pK_a of the SH group of each thiol. Results from three independent experiments are expressed as means±SD.

level of °OH production than GSH itself, and than other tested biological thiols (Fig. 6). This fact has a real biological interest because it was demonstrated that extracellular ROS production occurring during GSH catabolism participates to signal transduction [3] and cell component damages, especially lipid peroxidation [4,5].

The thiol drugs presently tested are active substances in rheumatoid arthritis {D-penicillamine, 2-mercaptopropionylglycine (MPG) and its main metabolite, 2-mercaptopropionic acid (2-MPA) [23]}, an angiotensin converting enzyme inhibitor, captopril (CSH), and an antidote for acetominophen poisoning, *N*-acetylcysteine (NAC).

High °OH production was only observed with racemic and pure enantiomeric forms of penicillamine. The other drugs exhibit low oxidant levels. 2-Mercaptoethanol (Merc) and 2,4-dithio-D,L-threitrol (DTT) with one or two sulfydryl groups, are frequently used as reducing reagents in biochemical studies. They exhibit opposite effects, very low and high °OH production levels, respectively.

The relative reactivity of thiols at pH 7.4, determined as their °OH production level is probably related to the pK_a of the sulfhydryl group (Fig. 6), as already mentioned [5,7,22]. The proposed mechanism indicates that the thiolate anion is important in at least one step of the reaction. Thus, lower the pK_a value is, higher is the proportion of thiolate and consequently the thiol reactivity increased. However, ionization is not the only determinant of their reactivity because no real correlation could be established between pK_a values and °OH production levels. Other factors especially for aminothiols (length of the hydrocarbon chain [24], complexation constant with ferric ion [25]) are participating in the observed variations in oxidant effect.

The °OH production process depending on the GGT activity was monitored in a cellular model (NIH3T3/GGTrel cDNA transfected cells) vs. NIH3T3 control cell line, using cell pellets in the presence of GSH and EDTA chelated Fe^{3+} (Fig. 7). The GGT-catalyzed reaction was performed in presence of glygly, which is an acceptor substrate and so increases the rate of the enzymatic reaction. The resulting data supply evidence for the proposed prooxidant mechanism of ROS production initiated by GGT enzyme in the presence of its natural

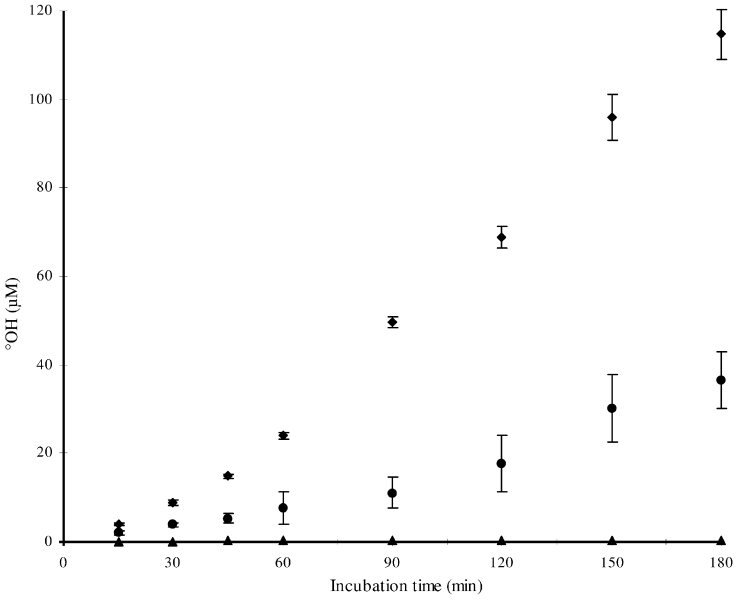

Fig. 7. Time course of °OH production monitored during GSH breakdown via GGT using salicylic acid (2 m*M*) hydroxylation. Incubation was realized in presence of 10^6 cell pellets expressing GGTrel (♦) or not (●), in presence of GSH (2.5 m*M*) or without (▲), glycylglycine (25 m*M*), Fe^{3+} 150 μ*M* and EDTA 165 μ*M* in PBS, pH 7.4 at 37°C. Results from three independent experiments are expressed as means±SD.

substrate GSH and chelated metals [7]. As previously suggested, these chain reactions involve the production of the powerful oxidant °OH and may account for lipid peroxidation reported by several studies [4,5].

The present technique is a helpful analytical tool to quantify °OH production during in vitro experiments involving thiols and is presently under investigation to monitor °OH during GSH metabolism in both extra- and intra-cellular media of different cultured cell lines in presence of various sources of metallic ions.

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