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Determination of hydroxyl free radical formation in human platelets using high-performance liquid chromatography with electrochemical detection

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Abstract

The formation of the hydroxyl free radical (HFR) can be quantified indirectly, by measuring two products of the hydroxylation of salicylic acid, 2,3-dihydroxybenzoate (2,3-DHB) and 2,5-dihydroxybenzoate (2,5-DHB). In this study, we used reversed-phase high-performance liquid chromatography with electrochemical (coulometric) detection to measure 2,3- and 2,5-DHB levels in human platelets. The limits of detection of the method were 10 and 5 fmol on column for 2,3-DHB and 2,5-DHB, respectively. We tested the technique by measuring increases in dihydroxybenzoate levels after exposure of platelets to experimentally induced oxidative stress. Then, we measured platelet levels of 2,3- and 2,5-DHB in patients with Parkinson's disease, under therapy with L-DOPA, and in normal subjects. We also measured platelet concentrations of L-DOPA and its major metabolite, 3-*O*-methyldopa (3-OMD). Parkinsonian patients showed increased levels of both 2,3- and 2,5-DHB. Platelet levels of 2,3-DHB were positively correlated with platelet levels of L-DOPA and 3-OMD. The technique we describe proved simple and extremely sensitive and may represent a useful tool for the study of oxidative stress in humans.

Keywords: Hydroxyl free radical formation; Dihydroxybenzoate; L-DOPA

1. Introduction

Reactive oxygen species (ROS) form continuously in the body, as a result of various biochemical processes. Due to the presence of an unpaired electron, ROS are highly unstable and tend to react with cellular constitutive elements, particularly lipids and nucleic acids, in order to stabilize. This reaction is oxidative in nature and accounts for the potential cytotoxicity of ROS [1]. Of all the ROS, the hydroxyl free radical (HFR) is considered the most reactive and hazardous. HFR ($^{\cdot}OH$) is extremely difficult to measure directly. Therefore, indirect methods have been developed to quantify its formation. One of these methods involves the reaction of salicylate with the HFR. Such a reaction generates two stable adducts, 2,3- and 2,5-dihydroxybenzoates (2,3-DHB and 2,5-DHB, respectively), which can be measured reliably with reversed-phase high-performance liquid chromatography (HPLC) and electro-

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chemical detection [2–4]. This method has been extensively used in animal research, to detect HFR formation under various experimental conditions that cause oxidative stress [5–11]. This technique has also been used in clinical research. Various authors have measured plasma levels of 2,3- and 2,5-DHBs after systemic administration of *O*-acetylsalicylic acid (aspirin) in humans. The technique has been used to quantify systemic oxidative stress, for example, in diabetic patients [12,13]. The method has also been applied to the determination of HFR generation in human platelets exposed to anoxia/reoxygenation [14]. Amperometric detection has been mostly used in these studies. Recently, McCabe et al. [4] described a method for the estimation of dihydroxybenzoates based on the use of reversed-phase HPLC with dual coulometric electrode detection.

Oxidative stress, resulting from increased formation and/or defective inactivation of ROS, might play an important role in the pathogenesis of neurodegenerative disorders, particularly in Parkinson's disease (PD) [15]. In PD patients, various markers of lipid peroxidation and oxidative damage to DNA are increased in the substantia nigra [16,17], which is the area of the brain selectively affected by the degenerative process. A deficiency of mitochondrial enzyme complex I — a condition that causes increased ROS formation — is also present in this area [18,19]. Peripheral signs of increased oxidative stress have also been reported in PD. Decreased activity of the antioxidant enzyme superoxide dismutase and increased susceptibility to lipid peroxidation have been found in the erythrocytes of PD patients [20,21]. It has also been reported that the ROS-producing activity of polymorphonuclear leukocytes and the plasma levels of malondialdehyde, a product of lipid membrane peroxidation, are increased in PD patients [22]. Furthermore, a reduction in the activity of complex I has been repeatedly found in platelets of PD patients [23–25].

An issue that is currently being debated is the potential toxicity of L-DOPA, the most used pharmacological treatment for PD. It has been hypothesized that L-DOPA may contribute paradoxically to progression of the disease. This would be a consequence of the auto-oxidative metabolism of the drug, which generates a variety of ROS [26,27]. In the rat, systemic administration of L-DOPA increases the production of ROS in the substantia nigra [28]; in vitro, L-DOPA causes cell-death in neuronal and non-neuronal cell cultures [28–31]. However, no direct evidence of L-DOPA toxicity has been found in PD patients [32,33]. Thus, whether L-DOPA, as it is used in the clinical practice, can exert toxic effects remains controversial [34].

The aim of our study was to obtain an in vitro technique that measures the formation of 2,3- and 2,5-DHBs in human platelets incubated with sodium salicylate, using reversed-phase HPLC with electrochemical (coulometric) detection. As mentioned above, platelets have been extensively used to study PD pathophysiology. We have previously shown that, in PD patients, L-DOPA enters platelets [35] and facilitates the binding of the toxin 1-methyl-4-phenylpyridinium (MPP^+) to platelet complex I [36]. Thus, after verifying the possibility of determining the formation of dihydroxybenzoates in platelets exposed to experimentally induced oxidative stress, we measured 2,3- and 2,5-DHB formation in isolated platelets from PD patients treated with L-DOPA and from normal subjects. In order to verify the existence of a correlation between the formation of dihydroxybenzoates and intra-platelet levels of the drug, we also measured the platelet levels of L-DOPA and its major metabolite, 3-*O*-methyldopa (3-OMD).

2. Experimental

2.1. Chemicals and reagents

Sodium salicylate, 2,3-DHB, 2,5-DHB, pyrogallol, dimethylsulfoxide (DMSO), L-DOPA, 3-OMD, octyl sulfate, KH_2PO_4, $NaHCO_3$, NaCl, KCl, *N*-[2-hydroxyethyl]piperazine-*N′*-[2-ethanesulfonic acid] (HEPES), NaH_2PO_4, ethylenediamine tetraacetic acid (EDTA), $MgCl_2$ and dextrose were purchased from Sigma (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS) was purchased from Bio-Rad (Richmond, CA, USA). Acetonitrile, of HPLC grade, was purchased from Carlo Erba (Milan, Italy). Stock solutions of sodium salicylate, 2,3-DHB, 2,5-DHB, L-DOPA and 3-OMD were prepared by dissolving each compound in 0.1 *M* $HClO_4$.

2.2. Chromatography

2.2.1. Dihydroxybenzoates

The chromatographic system consisted of a pump (1350 Softstart, Bio-Rad, Hercules, CA, USA) equipped with a manual Rheodyne injector (Rheodyne, Cotati, CA, USA), a reversed-phase DHBA-250 column (C_{18}, 5 μm, 250×3.0 mm I.D.; ESA, Bedford, MA, USA) and an electrochemical detector with a dual electrode analytical cell (Coulochem 5100A; ESA) Oxidizing potentials were set at +250 mV for the first electrode (E1), and at +750 mV for the second electrode (E2) [4]. Dihydroxybenzoates were detected at the first electrode. The mobile phase (pH 2.75) contained 20 m*M* NaH_2PO_4, 30 m*M* sodium citrate, 75 m*M* EDTA and 150 m*M* octylsulfate, mixed with 5% (v/v) acetonitrile.

2.2.2. L-DOPA and 3-OMD

The chromatographic system consisted of a pump (System Gold 116, Beckman, San Ramon, CA, USA) equipped with a C_{18} reversed-phase 70×4.6 mm I.D., 3μm column (Ultrasphere XL ODS, Beckman), fitted with a 5×4.6-mm pre-column (Ultrasphere XL ODS, Beckman) and connected to an autosampler (AS 100, Bio-Rad, Richmond, CA, USA). The detection device was an electrochemical detector (Coulochem 5100A, ESA), equipped with a conditioning cell, with one electrode set at +200 mV, and a dual electrode analytical cell. Potentials of the analytical electrodes were set at −200 mV (E1) and +300 mV (E2). L-DOPA and 3-OMD were detected at the second electrode [35]. The mobile phase (pH 2.9) consisted of 50 m*M* KH_2PO_4, 0.7 m*M* sodium dodecylsulphate and 0.3 m*M* EDTA, mixed with 12% (v/v) acetonitrile.

Data from the two systems were collected and integrated, through separate channels, by a dedicated PC equipped with chromatography software (Value Chrom, Bio-Rad, Hercules, CA, USA).

2.3. Sample preparation

Whole blood was drawn from the antecubital vein, without tourniquet, and collected into plastic tubes containing sodium citrate (Terumo Medical, Elkton, MD, USA). Blood was then centrifuged at low speed (200 *g*×15 min) to obtain platelet-rich plasma (PRP). PRP was separated and platelets were counted with an automated cell analyzer (Technicon H1, Technicon Instruments, Tarrytown, NY, USA). This also allowed us to verify the absence of any contamination from red blood cells or leukocytes. Aliquots of PRP (1 ml) were then incubated with sodium salicylate, in the dark (concentration of sodium salicylate and the duration of the incubation varied, according to the experimental conditions. See below). After incubation, platelets were pelleted by centrifugation at 2000 *g* for 15 min, washed with modified Tyrode's solution (7 m*M* NaHCO3, 150 m*M* NaCl, 2.7 m*M* KCl, 0.55 m*M* NaH2PO4, 1 m*M* EDTA, 0.5 m*M* MgCl2, 5.6 m*M* dextrose) and reconstituted through a 15-min centrifugation at 2000 *g*. Supernatants were then removed and platelet pellets were stored at −20°C until the chromatographic assays were performed (always within one week). Incubation and all centrifugations were carried out at room temperature.

Before the HPLC assay, platelet pellets were resuspended in 1 ml of ice-cold $HClO_4$ (0.4 *M*) and homogenized using ultrasounds (Ultrasonic 2000, Artek, Farmingdale, NY, USA). Since the sonication process itself can generate ROS [37,38], the influence of sonication on the formation of 2,3-DHB and 2,5-DHB was verified by sonicating pellets obtained from the same pool of PRP for 10, 30 and 60 s. The procedure was carried out in the presence and absence of 0.7 *M* DMSO, a nonspecific oxyradical scavenger. Homogenates were then centrifuged at 15 000 *g* for 5 min, at room temperature, and supernatants (50 μl) were directly injected into the HPLC system.

2.4. Experimental study

Blood was obtained from ten healthy volunteers (six males and four females, ranging in age between 25 and 35 years). PRP from all samples was pooled and aliquoted into 1-ml volumes. PRP samples were then incubated with increasing concentrations of sodium salicylate (2.5, 5, 10, 20 and 40 m*M*). Other PRP samples were incubated with 10 m*M* sodium salicylate for increasing periods of time (30 min, and

1, 2, 4 and 8 h). After incubation, samples were processed as described above.

After an appropriate concentration of sodium salicylate (10 m*M*) and incubation time (4 hours) were established, experiments were carried out to investigate how enhanced oxidative stress affects the conversion of sodium salicylate into 2,3-DHB and 2,5-DHB in platelets. For this purpose, blood was drawn from 15 healthy volunteers (eight males and seven females, ranging in age between 24 and 35 years) to obtain a pool of PRP that was subsequently aliquoted into 1-ml volumes. PRP aliquots were then incubated with sodium salicylate and 0.2 m*M* hydrogen peroxide. Before starting the incubation, PRP samples were exposed for 15 min to UV rays (310 nm; Bio-Rad, UV GEN™). This procedure has been shown to generate HFR, as a result of hydrogen peroxide photolysis [2]. In another experiment, 1-ml volumes of PRP were incubated with sodium salicylate and 0.5, 1 or 2 m*M* pyrogallol, a generator of free radical superoxide anion ($^{\cdot}O_2^-$), in the presence and absence of 0.7 *M* DMSO. After incubation, samples were processed as described above.

The precision of the method was evaluated by preparing 20 platelet pellets from a common pool of PRP that had been incubated previously with sodium salicylate (10 m*M*) for 4 h. Ten samples were then analyzed within one day (within-run precision) while the others were analyzed separately, on ten different days (between-run precision).

2.5. Clinical study

Twenty five PD patients under treatment with L-DOPA [12 males and 13 females, with a mean age of 61±1.5 years (SEM)] and 22 healthy volunteers, who were matched for age (59.1±1.6 years) and sex (ten males and 12 females) were enrolled. All patients had been previously diagnosed as having idiopathic PD, at the *Center for Parkinson's Disease and Movement Disorders* of the Neurological Institute ''C. Mondino'' of Pavia, and were being treated as outpatients at the time of enrollment. All of them were taking L-DOPA as monotherapy for PD. Patients taking medications other than L-DOPA were excluded from the study. The daily dose of L-DOPA ranged between 250 and 1825 mg (mean±SEM, 765±63). None of the subjects, either patients or controls, was taking salicylates at the time of the study.

All subjects underwent venipuncture between 10 and 11 a.m.: for treated PD patients, this corresponded to an interval of 3 to 4 h from the last dose of L-DOPA. Venous blood (10 ml) was collected following the procedure described above. Blood was then centrifuged at 200 *g* for 15 min to obtain PRP. PRP was separated and platelets were counted. Two 1-ml volumes of PRP from each subject were then separated and incubated with 10 m*M* sodium salicylate for determination of 2,3- and 2,5-DHB, as described above. The remaining PRP was used for the analysis of platelet levels of L-DOPA and 3-OMD (see Ref. [35] for details). All measurements were performed in duplicate.

2.6. Statistics

Comparisons between groups were made using the Student's *t*-test for unpaired observations. The existence of correlation between variables was investigated by calculating the Pearson's correlation coefficient (*r*). The minimum level of statistical significance was set at $P<0.05$.

3. Results and discussion

3.1. Chromatography

Oxidation of 2,3- and 2,5-DHBs was performed at the first electrode of the coulometric detector, using the conditions described by McCabe et al. [4]. Sodium salicylate was not measured. Chromatographic separation of the two compounds of interest was complete and was achieved within 6 min. The detector response to increasing concentrations of 2,3-DHB and 2,5-DHB was linear. Calibration curves were obtained by injecting increasing concentrations of 2,3-DHB and 2,5-DHB standards. Concentrations were 4, 8, 16, 32 and 64 pmol/ml for 2,3-DHB (curve equation: $y=0.67+2.03x$; $r^2=0.998$) and 2, 4, 8, 16 and 32 pmol/ml for 2,5-DHB (curve equation: $y=1.37+5.44x$; $r^2=0.997$). Detection limits, at a signal-to-noise ratio of three, were 10 and 5 fmol on column for 2,3-DHB and 2,5-DHB, respectively. The within-run and between-run coefficients of variation

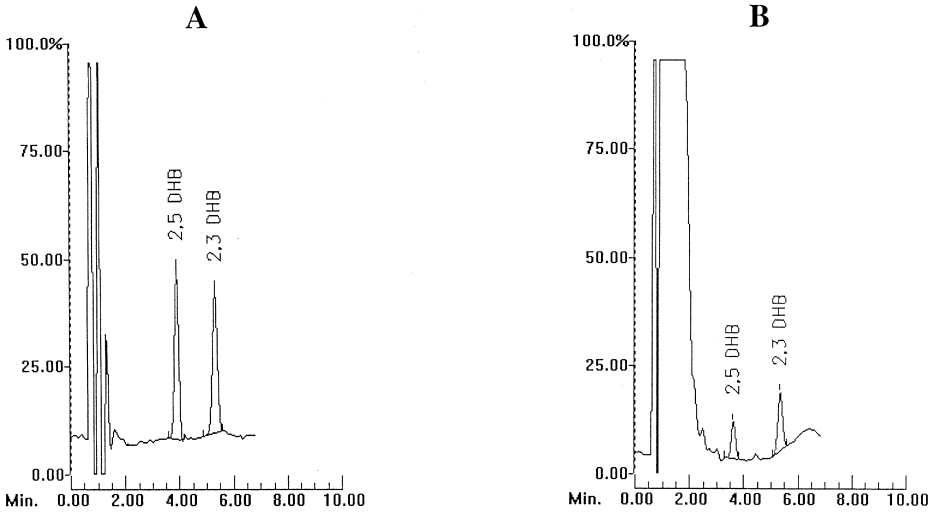

Fig. 1. Chromatograms of (A) a standard mixture containing 2,3-DHB (32 pmol/ml; 1.6 pmol injected) and 2,5-DHB (16 pmol/ml; 0.8 pmol injected), and (B) a platelet sample (2,3-DHB: 29 pmol/10^9 platelets, 2,5-DHB: 10 pmol/10^9 platelets).

were 12.2 and 14.8% for 2,3-DHB, and 13.1 and 16.6% for 2,5-DHB. Fig. 1 shows typical chromatograms of 2,3- and 2,5-DHB peaks in a standard mixture and in a platelet sample.

3.2. Experimental study

Incubation of samples with increasing concentrations of sodium salicylate caused platelet levels of 2,3- and 2,5-DHBs to increase linearly, until their values tended to reach a plateau for concentrations of sodium salicylate exceeding 10 m*M* (Fig. 2). The duration of the incubation of PRP with sodium salicylate also affected the formation of 2,3-DHB and 2,5-DHB, the maximum value being reached after 4 h of incubation.

We determined if our method was able to detect dihydroxybenzoate increases in platelets exposed to conditions of enhanced oxidative stress. Incubation of PRP samples with sodium salicylate and hydrogen peroxide, after exposure to UV rays, gave rise to massive increases in platelet levels of dihydroxybenzoates, particularly of 2,3-DHB (Fig. 3). These increases were effectively, although not completely, counteracted by the presence of a nonspecific free-radical scavenger such as DMSO. Far less marked increases in 2,3- and 2,5-DHB levels were observed with pyrogallol, and only when the highest dose (2 m*M*) was used (Fig. 3). In this case, the increases in dihydroxybenzoate concentrations were completely prevented by DMSO.

UV-induced photolysis of hydrogen peroxide causes the selective formation of HFR [2], while pyrogallol is a pro-oxidant agent that acts mainly through the generation of superoxide anion. Our findings show that experimentally induced oxidative stress causes increased intra-platelet formation of dihydroxybenzoates and confirm that sodium salicyl-

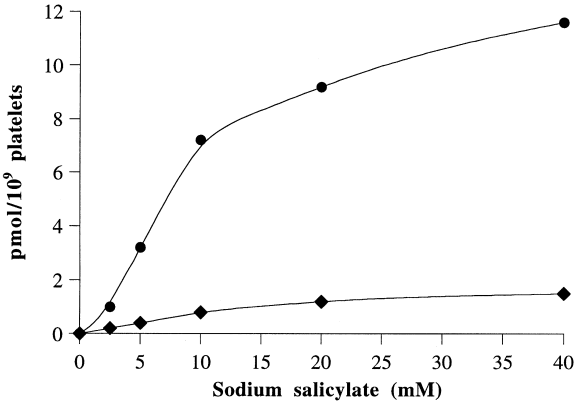

Fig. 2. Platelet levels of 2,3-DHB (–●–) and 2,5-DHB (–✦–) after incubation of platelet-rich plasma with increasing concentrations of sodium salicylate.

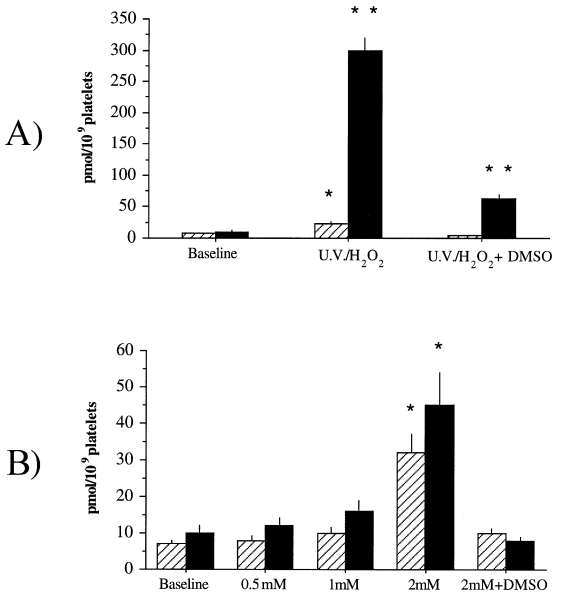

Fig. 3. (A) Platelet levels of 2,3-DHB (■) and 2,5-DHB (▨) after incubation of PRP samples with 10 m*M* sodium salicylate and 0.2 m*M* hydrogen peroxide (H_2O_2). Incubation was preceded by a 15-min irradiation with UV rays (310 nm); the experiment was carried out in the absence or presence of the oxyradical scavenger dimethylsulfoxide (DMSO). (B) Platelet levels of 2,3-DHB (■) and 2,5-DHB (▨) after incubation of PRP samples with 10 m*M* sodium salicylate and increasing concentrations (0.5, 1 and 2 m*M*) of pyrogallol. The experiment using the highest dose of pyrogallol (2 m*M*) was repeated in the presence of 0.7 *M* DMSO. Each bar represents the mean±SEM of five experiments; **P* <0.01, ***P*< 0.001 vs. baseline values (Student's *t*-test).

ate is a far more specific trapping agent for HFR than for superoxide anion [2–4].

Sonication is known to induce ROS formation [37,38]. Indeed, we observed that increases in the duration of pellet sonication (10–60 s) were paralleled by increases in the platelet levels of dihydroxybenzoates. This was probably due to the intraplatelet presence of residual amounts of sodium salicylate, which may have reacted with newly formed ROS. The addition of 50 μl of DMSO, immediately before sonication, caused a 60% decrease in the platelet levels of 2,3- and 2,5-dihydroxybenzoates observed at the first time-point (10 s, which was the minimum required to obtain satisfactory homogenization of pellets). More importantly, no significant increases were observed at the subsequent time-points. DMSO was then routinely added, before sonication, to all samples that were processed.

3.3. Clinical study

Blood platelets have been used extensively to explore various pathogenetic aspects of neurodegenerative disorders, particularly of PD [23–25,35]. In this study, we measured concentrations of 2,3- and 2,5-DHB in platelets of PD patients treated only with L-DOPA and from normal subjects, after incubation of PRPs with sodium salicylate. We also measured platelet levels of L-DOPA and its major metabolite, 3-OMD. The purpose was to determine if peripheral signs of enhanced oxidative stress, possibly related to the pharmacological treatment, can be detected in parkinsonian patients. PD patients showed higher levels of both 2,3-DHB (14.2±1.4 vs. 8.2±0.8 pmol/10^9 platelets; $P<0.05$) and 2,5-DHB (12.7±1.8 vs. 7.1±0.9 pmol/10^9 platelets; $P<0.01$) than controls. Furthermore, in PD patients, platelet levels of 2,3-DHB were positively correlated with platelet concentrations of both L-DOPA and 3-O-methyldopa (Fig. 4). No significant correlation was found between the levels of the drug or its metabolite and platelet levels of 2,5-DHB.

The absence of a group of untreated PD patients does not allow us to conclude whether such a phenomenon was related to the therapy or to the disease per se. However, the direct correlation between platelet levels of 2,3-DHB and both L-DOPA and 3-OMD suggests that the increased hydroxylation of salicylate may be, at least partially, related to the presence of the drug, as a result of its intracellular auto-oxidative metabolism [26–30]. Whether or not this phenomenon, which was modest in terms of absolute values, is relevant to the pathophysiology of PD remains to be established.

4. Conclusions

Previous HPLC methods used for clinical research have measured plasma dihydroxybenzoates after systemic administration of *O*-acetylsalicylic acid, thus providing an index of generalized HFR forma-

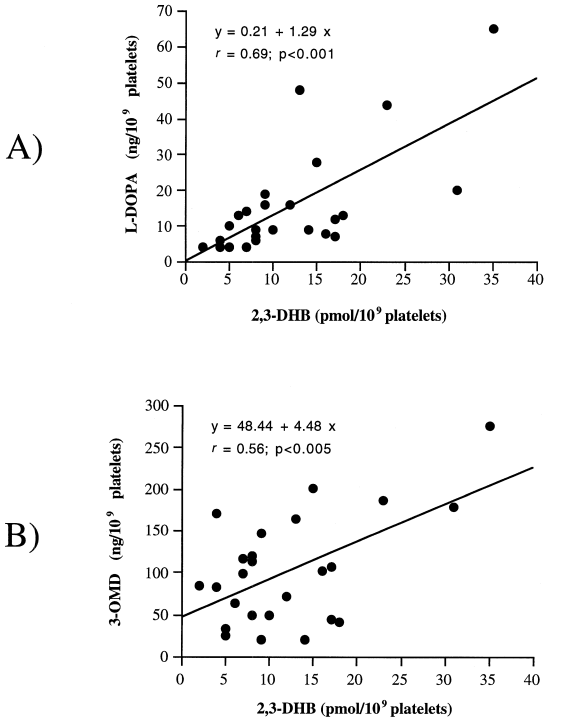

Fig. 4. Correlation between platelet levels of 2,3-DHB and (A) L-DOPA or (B) 3-*O*-methyldopa (3-OMD), in parkinsonian patients.

tion [12,13]. Our purpose was to focus the investigation on blood platelets, since these cells can intrinsically generate ROS [39]. Furthermore, by using an in vitro technique, we intended to avoid potential confounding factors related to the systemic administration of *O*-acetylsalicylic acid, such as, for example, inter-individual variability in the intestinal absorption rate of the drug. Therefore, we devised a method for measuring 2,3- and 2,5-DHB levels, which are two indirect indices of HFR formation, in human platelets incubated with the parent compound, sodium salicylate. The method, which proved simple and extremely sensitive, enabled the detection of intra-platelet formation of HFR under experimentally induced oxidative stress. Furthermore, this technique allowed us to detect an increase in the formation of 2,3- and 2,5-DHB levels in platelets of parkinsonian patients treated with L-DOPA, which may be related to the pro-oxidant properties ascribed to this drug.

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