Determination of ascorbic acid by isotachophoresis with regard to its potential in neuroblastoma therapy

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ABSTRACT

Analytical capillary isotachophoresis was used to determine ascorbic acid (AA) in different matrices (cell-free system, neuroblastoma cell extracts and urine). The system for purging bone marrow of neuroblastoma cells, including 6-hydroxydopamine (6-OHDA) and AA, was analysed with regard to the interaction of AA with 6-OHDA and its autoxidation product, hydrogen peroxide. Furthermore, analyses concerning the uptake of AA into neuroblastoma cells as well as its excretion in urine after uptake of large amounts were carried out.

INTRODUCTION

Neuroblastoma is a tumour of the sympathetic nervous system, which in its disseminated form has a poor prognosis [1]. One new therapeutic approach in the treatment of this tumour is autologous bone marrow transplantation. Since neuroblastoma cells metastasize into bone marrow, bone marrow cells have to be cleared of neuroblastoma cells prior to reinfusion. Among other substances, the neurotoxin 6-hydroxydopamine (6-OHDA) has been used as a purging agent [2]. It is supposed that since 6-OHDA is a catecholaminergic compound it is incorporated specifically into neuroblastoma but not into bone marrow stem cells. Inside the cells it can autoxidize, leading to cytotoxic oxygen compounds such as superoxidanion, hydrogen peroxide and hydroxyl radicals (Fig. 1). Ascorbic acid (AA), added in a ten-fold excess, has been shown to enhance the cytotoxic effects of 6-OHDA [3]. It acts a redox cycler and reduced 6-OHDA-chinon (which is formed during the oxidation of 6-OHDA [4]), allowing the next round of cycling to generate reactive oxygen compounds. However, as will be shown in this paper, some of the suppositions mentioned above have proved to be incorrect. This was demonstrated by investigating the interaction of AA with 6-OHDA and hydrogen peroxide using isotachophoresis (AA), HPLC (6-OHDA) and the Clark electrode (oxygen consumption).

EXPERIMENTAL

Chemicals

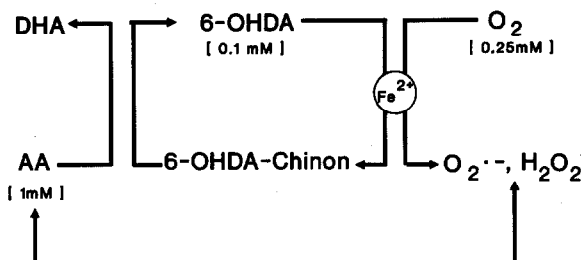6-OHDA, EDTA, 1,10-phenanthroline and

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Fig. 1. Redox cycling of 6-OHDA in the presence of excess AA. In the presence of oxygen and traces of iron, 6-OHDA oxidizes to 6-OHDA-chinon, leading to the formation of reactive oxygen compounds such as the superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). The molarities indicate the concentrations present in the bone marrow-purging system. DHA = Dehydroascorbic acid.

ascorbic acid were from Sigma (Munich, Germany); desferrioxamine (Desferal, DFO) was obtained from Ciba (Basle, Switzerland).

Isotachophoresis

Experiments were performed on a LKB Tachophor 2127 equipped with UV (254 nm) and conductivity detectors. 0.01 M HCl adjusted with β-alanine to pH 3.65 plus 0.3% (w/v) methylcellulose 4000 (Fluka, Buchs, Switzerland) was used as the leading electrolyte and 0.01 M caproic acid (Merck, Darmstadt, Germany) as the terminating electrolyte. Analysis was carried out at 15°C in a 230 × 0.5 mm I.D. capillary. Usually, the current during signal detection was 7.5 μA. Chart speed during detection was 10 cm/min.

Other methods

Oxygen consumption during autoxidation of 6-OHDA experiments was measured with a Clark-type electrode (Hansa-Tech, UK, [5]).

HPLC (electrochemical detection) was used for the determination of 6-OHDA.

Cell culture

The human neuroblastoma cell line SK-N-SH was cultivated in RPMI (Rosewell Park Memorial Institute) 1640 cell culture medium supplemented with 2 mM L-glutamine, penicillin/streptomycin and 10% foetal calf serum. AA was added in a final concentration of 1 mM. After different incubation times, cell culture medium was removed and cell pellets were extensively

washed and lysed in 50 μl of 0.1 M hydrochloric acid. A 5-μl volume was injected into the Tachophor.

RESULTS AND DISCUSSION

Identification of asorbic acid in different matrices

Fig. 2 shows isotachopherograms of AA in different matrices [phosphate-buffered saline (Fig. 2a) cell lysates of the neuroblastoma cell
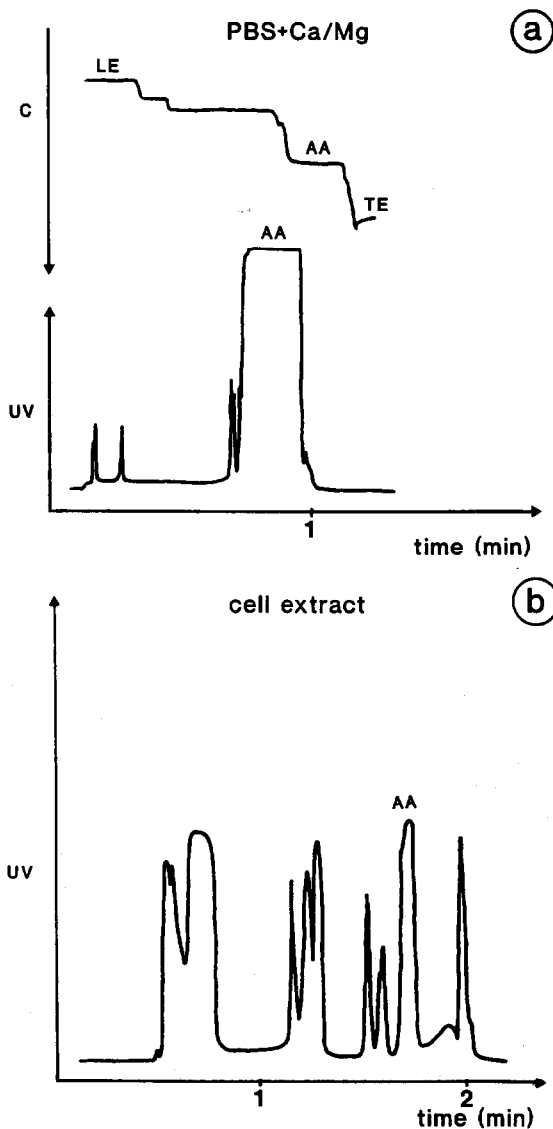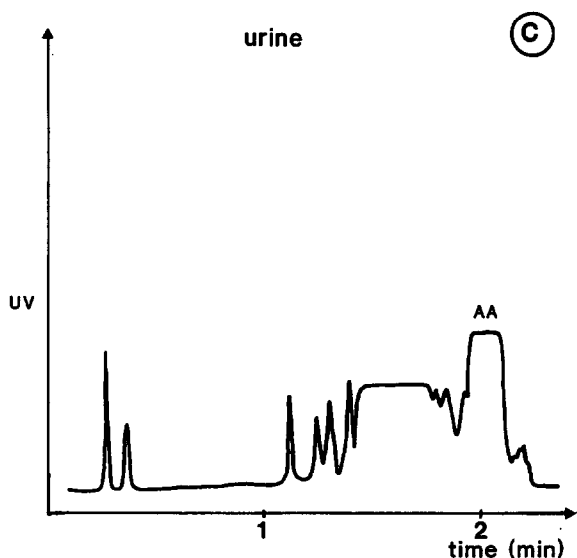

Fig. 2.

Fig. 2. Isotachopherograms of AA in different biological matrices (UV, UV signal, 254 nm; C, conductivity). (a) AA 5 mM in phosphate-buffered saline. (b) AA in extracts of $3.6 \cdot 10^6$ SK-N-SH cells after 4 h incubation with 1 mM ascorbate. (c) AA in 1:1 diluted morning urine after uptake of 1 g of ascorbic acid the night before. LE = Leading electrolyte; TE = terminating electrolyte.

line SK-N-SH (Fig. 2b) and 1:1 diluted urine (Fig. 2c)]. AA was identified by its characteristic UV pattern at 254 nm, its conductivity signal, and by addition of AA to neuroblastoma cell extracts and urine. Calibration curves were linear in all three matrices (data not shown).

Interaction of 0.1 mM 6-OHDA with 1 mM ascorbic acid

The combination of 0.1 mM 6-OHDA and 1 mM AA has been used clinically to purge bone marrow of neuroblastoma cells. The kinetics of 6-OHDA autoxidation and its interaction with AA was investigated and is shown in Fig. 3a–c. Within 15 mins, about 70% of 6-OHDA was metabolized in the presence of AA (Fig. 3a). Although present in a tenfold molar excess compared with 6-OHDA, only 18% of AA was consumed during this period of time (Fig. 3b) and 37% after 60 mins. It was expected that in the presence of a tenfold excess of ascorbic acid several redox cycles would be possible. However, the limiting factor for redox cycling proved

to be not ascorbic acid but the oxygen concentration in the reaction mixture (about 250 μmol/l [4], Fig. 3c, compared to 100 μM 6-OHDA and 1000 μM AA). Therefore, even with a tenfold excess of ascorbate compared with
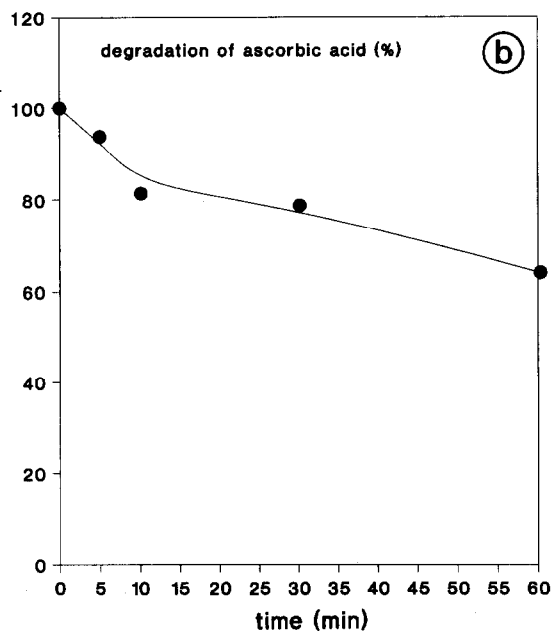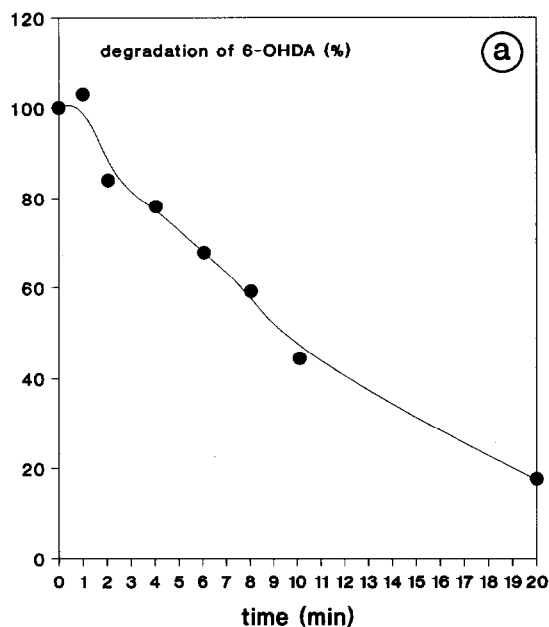

Fig. 3.   (Continued on p. 238)
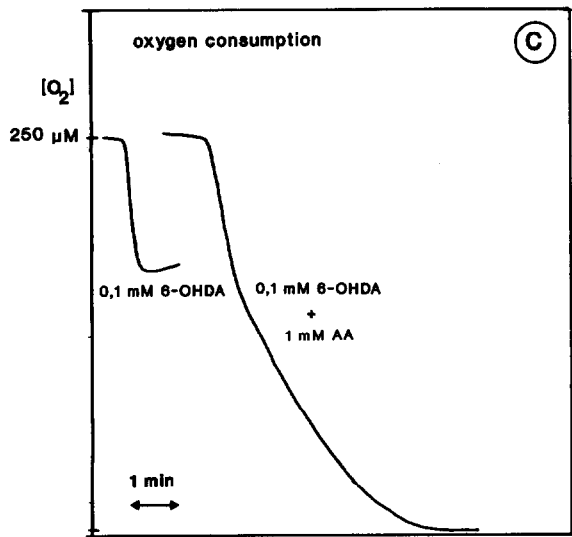
Fig. 3. Kinetics of the interaction between 0.1 mM 6-OHDA and 1 mM AA. (a) Time course of degradation of 6-OHDA (HPLC analysis). (b) Decrease of ascorbic acid (isotachophoresis). (c) Oxygen consumption (total sample volume: 1 ml).

6-OHDA, only about 1.5-fold redox cycling is possible until oxygen is completely consumed.

Nevertheless, after complete consumption of oxygen the tenfold excess of AA did not reduce, but even further enhanced, the toxic effects of the reaction mixture on neuroblastoma cells [3]. The major explanation is its interaction with hydrogen peroxide, which is formed during

TABLE I

DECREASE IN ASCORBIC ACID AFTER 30 min INCUBATION WITH HYDROGEN PEROXIDE[a]

AA (mM)	Hydrogen peroxide (mM)	Zone length of AA signal (mm)	Percentage of control
1	–	22.0	100
1	0.1	19.9	90.4
1	0.25	18.3	83.3
1	1	9.5	43.0

[a] AA, 1 mM, was incubated with different concentrations of hydrogen peroxide (0.1–1 mM) in 1 ml of phosphate-buffered saline, pH 7.4 at 37°C for 30 min. Subsequently 5 μl of the reaction mixture were injected into the Tachophor.

6-OHDA oxidation (Table I). The cytotoxic effects of AA are probably due to the formation of highly cytotoxic hydroxyl radicals in the Fenton reaction [6]:

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^\bullet + OH^-$$
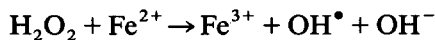
AA is able to reduce Fe^{3+} to Fe^{2+}, thus allowing this reaction to proceed. The important role of iron in the interaction of AA with hydrogen peroxide is documented in Fig. 4. The degradation rate of AA in the presence of hydrogen peroxide under different conditions was enhanced in the presence of additional iron, but reduced in the presence of the iron chelators Desferal and 1,10-phenanthroline. These experiments indicate that traces of iron, which are
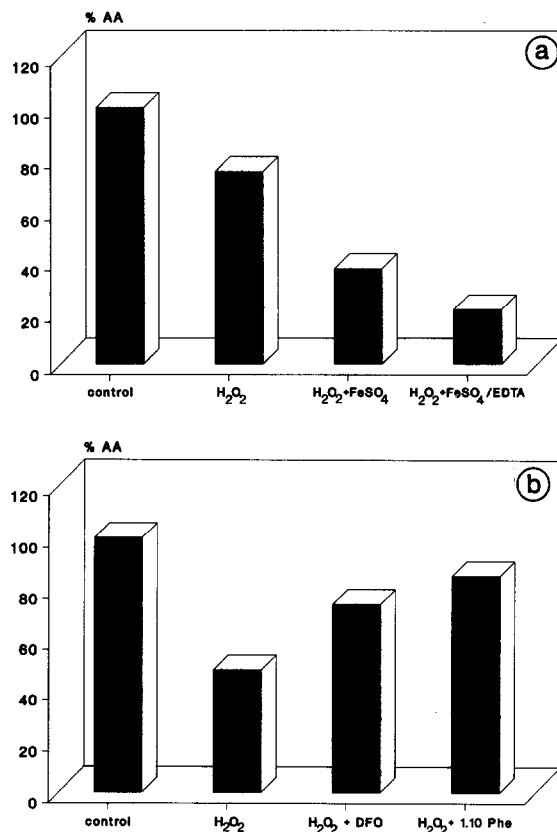

Fig. 4. Role of iron in the degradation of AA in the presence of hydrogen peroxide. (a) In the presence of elevated iron concentrations (AA, 10 mM; hydrogen peroxide 10 mM). (b) In the presence of the iron chelators Desferal (DFO) and 1,10-phenanthroline (1.10 Phe) (each 5 mmol/l): AA, 2.5 mM; hydrogen peroxide, 1 mM.

normally present in the reaction mixture, enable the Fenton reaction to occur.

The biological significance of this reaction is given by the observation that neuroblastoma cells endogenously generate hydrogen peroxide in elevated amounts [7], and that they contain elevated levels of ferritin from which iron can be released by AA [8,9]. Since AA is enriched in neuroblastoma cells in a time-dependent manner, the cytotoxic hydroxyl radicals could be formed (0.71 nmol of AA were found in 10^6 SK-N-SH cells after incubation with 1 mM ascorbic acid for 1 h, 2.5 nmol after 4 hs; Fig. 2b).

CONCLUSION

The present study shows that some of the ideas concerning the specific cytotoxicity of 6-OHDA on neuroblastoma cells in the presence of a tenfold excess of AA are not correct. The main limitation in using 6-OHDA/AA as a bone marrow-purging system is the fast capacity of 6-OHDA to autoxidize at neutral pH. Most of its

autoxidation product, hydrogen peroxide, is already formed outside the neuroblastoma cells before significant amounts of 6-OHDA can enter the cells. Furthermore, oxygen, not AA, is the limiting factor for the generation of reactive oxygen substrates during redox cycling. Nevertheless, the mixture of 6-OHDA and ascorbate proved to be more toxic the more ascorbate was present, probably because of the interaction of hydrogen peroxide and ascorbate, which, in the presence of traces of iron, leads to the formation of hydroxyl radicals.

Therefore, 6-OHDA/AA cannot be recommended as a bone marrow-purging agent. In addition, another conclusion concerning the therapeutic use of AA may be drawn from these experiments. Since neuroblastoma cells produce elevated amounts of hydrogen peroxide, and since they contain elevated amounts of ferritin from which iron can be released by ascorbic acid, daily application of large amounts of this vitamin to patients suffering from neuroblastoma may be a useful therapeutic approach for destruction of the tumour cells. Although ascorbate is excreted in large amounts in urine after its uptake in the gram range (Figs. 2c and 5), an increasing amount of it will accumulate in the body if it is given for an unlimited period of time. Continuous application of ascorbate may therefore be a mild therapy that could help destroy neuroblastoma cells.
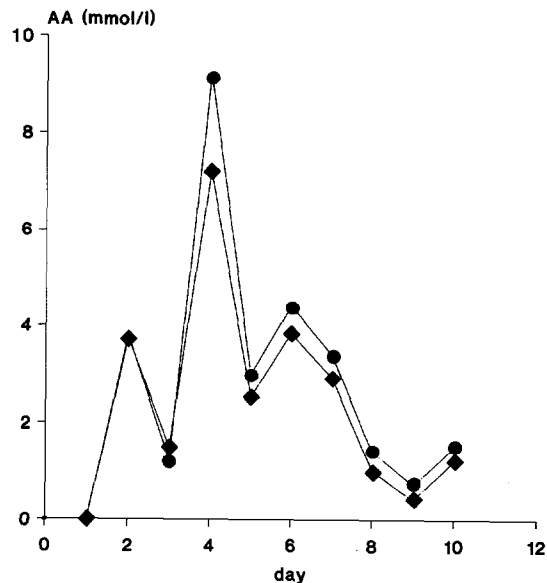

Fig. 5. Ascorbate concentration in morning urine after daily uptake of 1 g for 7 days (day 1: before uptake). AA in urine was determined in 1:1 diluted urine by isotachophoresis (●) and by a commercially available test [◆, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test, Böhringer Mannheim]. Correlation between both methods: $r^2 = 0.99$.

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