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Vitamin C and 6-amino-vitamin C conjugates of diclofenac: synthesis and evaluation

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Abstract

Diclofenac (Diclo), its ascorbic acid (AA) or 6-amino-AA (AA-NH_2) pro-drugs (AA-Diclo or AA-NH-Diclo) were prepared and evaluated on human retinal pigment epithelium (HRPE) cells to investigate their ability to interact with the vitamin C transporter SVCT2 and their cellular uptake. Furthermore, stabilities in physiological fluids of these compounds were investigated. For kinetic experiments, AA-Diclo was incubated in Tris–HCl buffer, human plasma or whole blood. The extracted samples were analysed by HPLC. AA-Diclo was hydrolysed following first order kinetics in buffer, plasma ($t_{1/2}$ about 10 h) and whole blood ($t_{1/2}$ about 3.5 h). Transport and inhibition assays were performed by adding [^{14}C]AA and the above-mentioned unlabelled compounds to plated HRPE cells. Intracellular accumulation was measured incubating HRPE cells with increasing concentrations of unlabelled compounds, following by HPLC analysis. Diclo resulted as a non-competitive inhibitor of AA-transport, showing a Na^+-dependent and ascorbate-independent uptake. AA-Diclo behaved as a competitive inhibitor, but it was not transported into cells, whereas its analogue AA-NH-Diclo showed a decreased inhibitory activity. Stability studies suggest AA-Diclo as a potential candidate to enhance the Diclo short half life in vivo. The discovery of a Na^+-dependent transporter for Diclo on HRPE cells opens new perspectives for targeting diclofenac into the brain.

Keywords: Diclofenac; Ascorbic acid; Pro-drug; SVCT2; Synthesis; Stability

1. Introduction

Diclofenac (Diclo, Fig. 1) is a non-steroidal anti-inflammatory drug, with analgesic and antipyretic activity, commonly used for the treatment of pain, fever and inflammation (Todd and Sorkin, 1988; Sharf et al.,

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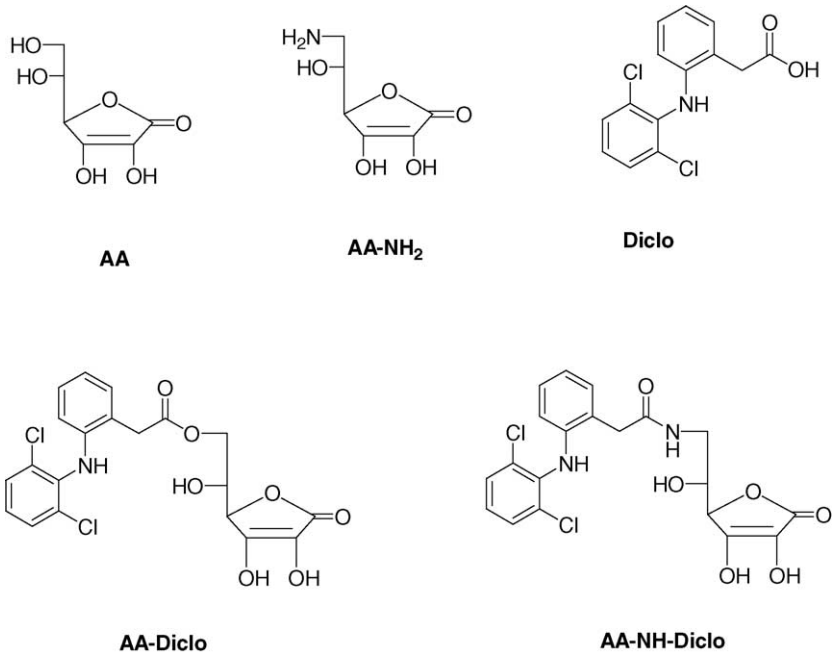


Fig. 1. Structures of ascorbic acid (AA), 6-amino-ascorbate (AA-NH_2), diclofenamic acid (Diclo) and their conjugates (AA-Diclo and AA-NH-Diclo).

1999). Recently, Diclo has been also investigated for its possible application to Alzheimer's disease, since long-term administration of anti-inflammatory drugs seems to have a protective effect on the onset of this pathology (McGeer et al., 1996; Halliday and Robinson, 2000; Ferencik et al., 2001). On the other hand, some properties of Diclo may be in contrast with an efficient therapy. As an example, it has been demonstrated that after intravenous administration to healthy subjects, Diclo shows a relatively low half life, near to 1 h (Todd and Sorkin, 1988; Sharf et al., 1999). Moreover, it is known that serious gastrointestinal problems can accompany the therapeutic effects of the non-steroidal anti-inflammatory drugs (Sharf et al., 1999; Halliday and Robinson, 2000).

In order to evaluate the behaviour of Diclo pro-drugs in physiological fluids and to investigate a potentially new way for its targeting into the central nervous system, we have studied the conjugation of Diclo with ascorbic acid (AA) and its 6-amino derivative (AA-NH_2), as shown in Fig. 1. In particular, the hydrolysis rate of the AA-Diclo conjugate (Fig. 1) has been evaluated in human plasma and whole blood, in order to explore a possible sustained release associated to potential reduction of the intensity of adverse effects. Moreover, taking into account that a new class of AA transporters (SVCT2, localized in the choroid plexus) has been recently characterized (Tsukaguchi et al., 1999; Rice, 2000) we have analysed the interaction of Diclo, AA-Diclo and AA-NH-Diclo (Fig. 1) with the SVCT2 transporters in order to get a better insight into the mechanism of transporters recognition. The measurements have been performed using a cellular model (human retinal pigment epithelium – HRPE – cells) previously developed by us (Manfredini et al., 2002). This study allows to investigate on a new approach considering the drug conjugation with AA or its derivatives as a potential mean of drug transport in the brain (Dalpiaz et al., 2004)

2. Materials and methods

[^{14}C]Ascorbate ([^{14}C]AA, 6 mCi/mmol) was obtained from NEN Life Science (Boston, MA, USA). Ascorbate (AA), dithiothreitol (DTT) and CHAPS

were obtained from Sigma (St. Louis, MO, USA). The conjugate AA-Diclo was synthesized as previously described (Manfredini et al., 2002). 5′-Octanoyl-N^6-cyclopentyladenosine (Oct-CPA) was prepared as previously described (Dalpiaz et al., 2001). HRPE cells (Rajan et al., 1999) were a kind gift of Prof. Puttur Prasad (Department of Obstetrics and Gynecology, Medical College of Georgia, Augusta, Georgia). FBS (foetal bovine serum), 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium, streptomycin and penicillin, trypsin-EDTA and PBS (phosphate-buffered saline) were obtained from Invitrogen (Life Technologies Italia, Milan, Italy)

HPLC grade solvents were purchased from Carlo Erba Reagenti (Milan, Italy). All other chemicals and solvents were obtained from standard sources.

2.1. Kinetic experiments

AA-Diclo was incubated at 37 °C in Tris–HCl buffer (100 mM, pH 7.4), human fresh plasma or whole blood obtained from healthy subjects. Three millilitres of buffer, plasma or whole blood were spiked with drug solutions resulting in final concentrations of 75 μM. At regular time intervals 100 μl of samples were withdrawn. Buffer samples (40 μl) were immediately injected in HPLC apparatus. Plasma or whole blood samples were quenched in 200 μl ice-cold ethanol or 500 μl ice-cold water, respectively. Fifty microlitres of 5×10^{-5} M internal standard (Oct-CPA) were added.

After 5 min of centrifugation at 9000 × *g*, 250 μl of plasma samples were reduced to dryness under a nitrogen stream. Two hundred microlitres of mobile phase were added and, after centrifugation, 40 μl were injected into HPLC system. Fifty microlitres of 10% sulfosalicylic acid were added to blood samples which were extracted twice with 1 ml water-saturated ethyl acetate. After 5 min of centrifugation at 9000 × *g*, the organic layer was reduced to dryness under a nitrogen stream. The residue was dissolved in 200 μl of mobile phase and 40 μl were injected into the HPLC system (see below). The half life of AA-Diclo hydrolysis was calculated from the analysis of the exponential increase of Diclo concentrations during time. The values were confirmed by semilogarithimic plots of differences, versus time, between the maximum Diclo amount obtained and the time increasing Diclo concentrations. Diclo peak areas or peak area ratios between the compound and internal standard were used, respectively, for buffer or plasma and blood samples.

2.2. HPLC analysis

The HPLC apparatus consisted of a modular chromatographic system (model 1100 series pump and diode array detector; Agilent, Waldbronn, Germany) linked to an injection valve with 50 μl sample loop (Model 9125; Rheodyne, Cotati, CA, USA). The detector was set at 266 nm. Chromatography of Diclo was performed on a reversed-phase column (LUNA CN C-18 5 μm, 150 mm × 4.6 mm i.d.; Phenomenex, Chemtek, Bologna, Italy), whereas chromatography of AA-Diclo was performed on a reversed-phase column (Hypersil BDS C-18 5 μm cartridge column, 150 mm × 4.6 mm i.d.; Alltech Italia Srl BV, Milan, Italy) equipped with a guard column packed with Hypersil C-18 material (Alltech). All analyses were performed at room temperature. Data acquisition and processing were accomplished with a personal computer using Chem Station software (Agilent). The mobile phase consisted of a mixture of acetonitrile and 50 mM phosphate buffer (pH 3.0) with a ratio of 60/40 (v/v). The flow rate was 0.8 ml/min. The retention times of Diclo and AA-Diclo were 3.1 and 3.5 min, respectively. The retention time of internal standard Oct-CPA was 4.8 min.

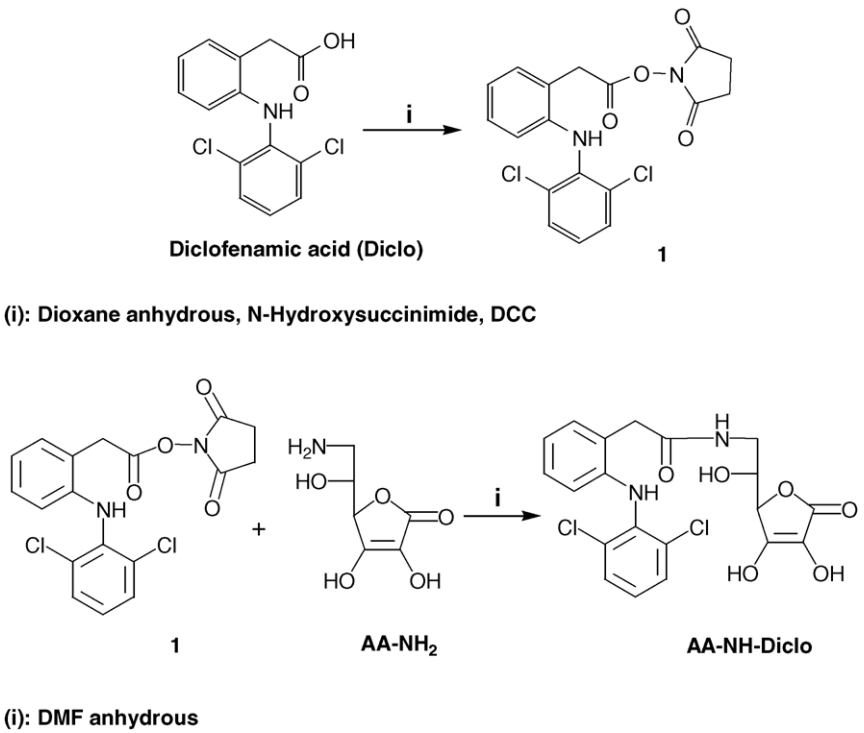
The identity peaks was assigned by co-chromatography with the authentic standards. Quantification was performed by integration of peak areas using external standardization.

2.3. Chemistry

The preparation of 5-(2-amino-1-hydroxy-ethyl)-3,4-dihydroxy-5*H*-furan-2-one (AA-NH_2; Scheme 1) was conducted as described in literature (Andrews, 1984).

2.3.1. Preparation of [2-(2,6-dichloro-phenylamino)-phenyl]-acetic acid 2,5-dioxo-pyrrolidin-1-yl ester (**1**)

To a solution of Diclo (296 mg, 1 mmol) in anhydrous dioxane (13 ml) hydroxysuccinimide was added (126.6 mg, 1.1 mmol) followed, at 0 °C, by DCC (227 mg, 1.1 mmol) also dissolved in anhydrous diox-



Scheme 1.

ane (10 ml) (Scheme 1). The reaction mixture was stirred at ambient temperature and under argon atmosphere for 12 h. After this time TLC (hexane/ethyl acetate, 7:3) indicated almost complete reaction, thus the formed DCU was filtered and the solution evaporated under vacuum. Compound **1** was then crystallized from isopropanol to give 370 mg of a white solid, yield 94%, mp 140–142 °C.

^{1}H NMR ($CDCl_3$): δ 2.84 (s, 4H, NHS), 4.13 (s, 2H, CH_2-phenyl), 6.2 (s broad, 1H, NH), 6.59–6.64 (dd, J = 7.9 Hz, 1H, C3′ phenyl), 6.93–7.35 (m, 6H, phenyl). MALDI-TOF MS: m/z 350.1 Da $(M + Na)^+$; 366.1 Da $(M + K)^+$.

2.3.2. Preparation of 2-[2-(2,6-dichloro-phenylamino)-phenyl]-N-[2-(3,4-dihydroxy-5-oxo-2,5-dihydro-furan-2-yl)-2-hydroxy-ethyl]-acetamide (AA-NH-Diclo)

Compound **1** (355 mg, 1 mmol) was added to a DMF solution containing (175 mg, 1 mmol) of AA-NH_2 (Scheme 1). After 4 h under stirring, at ambient temperature under argon atmosphere, the solvent was evaporated and the crude product was purified by column chromatography (CH_2Cl_2/MeOH gradient 9.8:0.2 to 8:2) to give 49.8 mg of a yellow solid, yield 11%, mp 139–142° C.

^{1}H NMR ($CDCl_3$): δ 3.21–3.48 (m, 2H hydroxy-ethyl C1-H); 3.70 (s, 2H, CH_2-phenyl), 3.88–4.04 (m, 1H, hydroxy-ethyl C2-H), 4.54 (d, J = 2.2 Hz, ascorbate C2-H), 5.02–5.20 (m, 4H, CH_2-phenyl), 6.43–6.52 (dd, 1H, J = 8.01 Hz, J = 1 Hz phenyl-acetyl H3), 6.54–7.40 (m, 17H, phenyl and NH-phenyl). MALDI-TOF MS: m/z 634.4 Da $(M + H)^+$; 656.5 Da $(M + Na)^+$; 672.9 Da $(M + K)^+$.

2.4. HRPE cell culture

HRPE cell line was routinely grown in 1:1 mixture of Dulbecco's modified Eagle's and Ham's F12 media, supplemented with 10% FBS, 50 μg/ml streptomycin and 50 IU/ml penicillin at 37 °C in 5% CO_2. Cells employed for uptake measurements were seeded in 24-well tissue culture plates and grown to confluence (2–3 days).

2.5. SVCT2 transporter interactions

Transport assays were performed using an uptake buffer prepared fresh each time, the composition of which was 25 mM Hepes/Tris (pH 7.5), 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, 1 mM DTT. Incubation time for the transport measurements was 60 min at 37° C, after this time the uptake buffer containing the radioactive substrate was aspirated of and cells were washed with 2× 2 ml of ice-cold uptake buffer. Cells were then solubilized in 250 μl of 0.2 M NaOH solution containing 0.5% CHAPS, transferred to vials and radioactivity associated with the cells was evaluated by liquid scintillation spectrometry.

The kinetics of [^{14}C]AA uptake, mediated by SVCT2, was analysed using concentration of AA ranging from 2.5 to 1000 μM. The concentration of [^{14}C]AA ranged from 2.5 to 50 μM and was kept constant at 50 μM. Data were analysed by nonlinear regression of Michaelis–Menten equation and confirmed by Eadie–Hofstee linear regression.

Inhibition of AA transport was determined by adding the indicated concentrations of unlabelled compounds to plated cells along with either [^{14}C]AA at fixed concentration 50 μM, or [^{14}C]AA ranging from 2.5 to 100 μM. The unlabelled inhibitor concentrations displacing 50% of [^{14}C]AA (IC_{50} values) were obtained by computer analysis of displacement curves. Inhibitory binding constants (K_i values) were derived from the IC_{50} values according to the Cheng and Prusoff equation $K_i = IC_{50}/(1 + [C^*]/K_t^*)$, where $[C^*]$ is the concentration of the [^{14}C]AA and K_t^* is Michaelis–Menten constant (Cheng and Prusoff, 1973). All calculations were performed using the computer program Graph Pad Prism (GraphPad).

Statistical analysis was performed by ANOVA followed by Dunnett's *t*-test. Difference was considered statistically significant at *P* values less than 0.05.

2.6. Uptake analysis in HRPE cells

Intracellular accumulation of Diclo and AA-Diclo were measured incubating HRPE cells with increasing concentrations of unlabelled analogue in uptake buffer for 60 min at 37 °C. Following the incubation, cells were washed with the uptake buffer and then lysed by adding deionized water (0.3 ml per well of a 24-well plate) and freezing the cells at −80 °C for 30 min. The cells were then be thawed on ice, and the lysate was centrifuged (12,500 × *g*—10 min) to remove cellular membranes. The supernatant (40 μl) was used to measure the levels of the test substrate by HPLC.

3. Results

The hydrolysis of AA-Diclo has been analysed in physiological fluids with the aim to investigate the stability of this Diclo pro-drug and, therefore, evaluate the opportunity to achieve the sustained release of the drug. Fig. 2 shows that the presence of AA-Diclo in

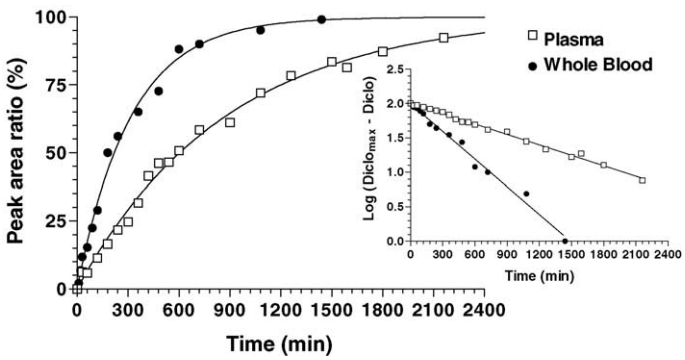


Fig. 2. Time course of diclofenamic acid (Diclo) obtained by AA-Diclo hydrolysis in human plasma and whole blood. Data are expressed as percentage of the peak area ratio referred to Diclo and the internal standard. The inset shows the semilogarithmic plots of differences, vs. time, between the maximum Diclo amount ($Diclo_{max}$) released and the time increasing Diclo amounts.

Table 1
Half-life values referred to the release of diclofenamic acid obtained by the hydrolysis of its ascorbyl conjugate (AA-Diclo) in buffer (pH 7.4), human plasma and human whole blood

Incubation condition	Half life (min)
Tris–HCl buffer	634 ± 58
Human plasma	602 ± 52
Human whole blood	222 ± 18

human plasma or human whole blood allows to obtain increasing concentrations of Diclo during time. The increase of the drug appears hyperbolically related to time; in these fluids no degradation of Diclo has been registered (data not shown). The semilogarithmic plots, reporting the AA-Diclo hydrolysis pattern, appear linear both in plasma ($n = 21$, $r = 0.997$; $P < 0.0001$) and in whole blood ($n = 15$, $r = 0.995$; $P < 0.0001$), indicating a first order kinetics of the pro-drug degradation. The different slopes of the plots indicate that the hydrolysis rates are different in plasma and human whole blood. In particular, as reported in Table 1, the half life of the pro-drug in whole blood is around 3.5 h whereas in plasma it is around 10 h. This last value is statistically comparable with the pro-drug hydrolysis in buffer.

The preparation of the amino-ascorbic-acid conjugate (AA-NH-Diclo) was first attempted by direct condensation between AA-NH_2 and Diclo, but unsuccessfully due to the poor reactivity of the latter acid. To overcome the problem Diclo was first activated by conversion into the corresponding *N*-hydroxysuccinimide derivative **1**, and then reacted with AA-NH_2 in DMF in presence of DCC, to give a complex reaction mixture that, after work-up and careful purification by column chromatography, gave the final conjugate AA-NH-Diclo in 11% yield (Scheme 1). The amount obtained was sufficient to the study purposes and thus the preparation was not optimized.

Table 2
Inhibition constant values (K_i) of ascorbic acid (AA), 6-amino-ascorbate (AA-NH_2), diclofenamic acid (Diclo) and their conjugates (AA-Diclo and AA-NH-Diclo) obtained by inhibition of 50 μM [^{14}C]AA uptake on HRPE cells

Compound	K_i (μM)
AA	20.1 ± 1.6
AA-NH_2	>1000
Diclo	3.35 ± 0.16
AA-Diclo	0.19 ± 0.01
AA-NH-Diclo	23.5 ± 1.8

The ability of Diclo and AA derivatives to interact with SVCT2 transporters of vitamin C has been analysed performing inhibition experiments of [^{14}C]AA uptake into HRPE cells, shown in Fig. 3. Table 2 reports the inhibition constant values (K_i) obtained by the analysis of the data depicted in Fig. 3. Diclo appears able to interact with the SVCT2 transporter ($K_i = 3.35 \pm 0.16$ μM) with higher affinity than the reference compound (ascorbic acid, $K_i = 20.1 \pm 1.6$ μM). Moreover, the conjugation of Diclo with AA allows to sensibly increase its SVCT2 affinity (AA-Diclo, $K_i = 0.19 \pm 0.01$ μM). On the other hand, the substitution of the hydroxyl moiety in position 6 of AA with an amino group (AA-NH_2), appears detrimental for the SVCT2 affinity. In fact, as shown in Fig. 3, the 6-amino-ascorbate derivative (AA-NH_2) is not able to interact with this transporter ($K_i \gg 1000$ μM). Moreover, it can

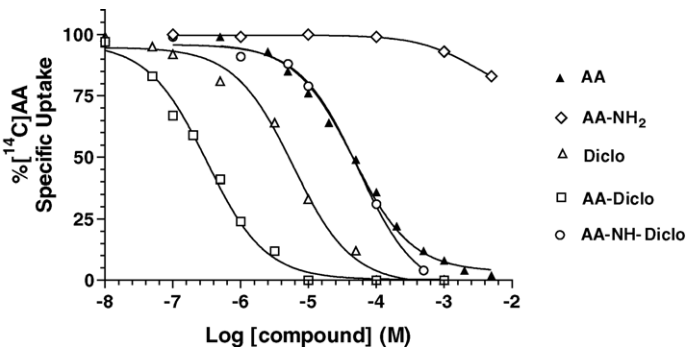


Fig. 3. Inhibition of 50 μM [^{14}C]AA uptake into HRPE cells by ascorbic acid (AA), 6-amino-ascorbate (AA-NH_2), diclofenamic acid (Diclo) and their conjugates (AA-Diclo and AA-NH-Diclo).

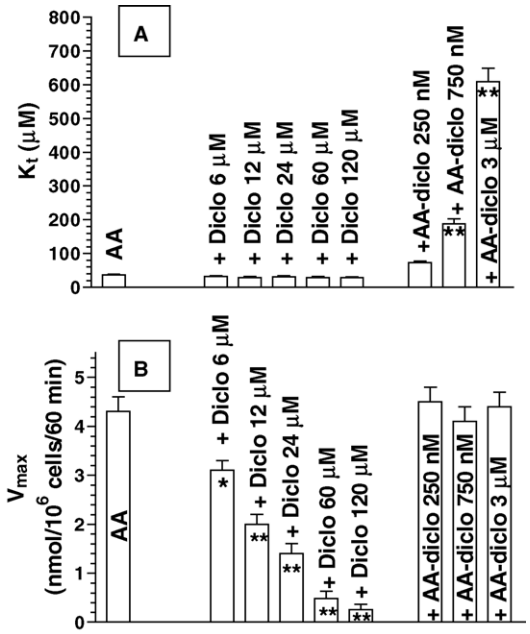


Fig. 4. K_t (A) and V_{max} (B) values of ascorbate transport obtained in the absence and in the presence of increasing concentrations of diclofenamic acid (Diclo) and its conjugate with ascorbic acid (AA-Diclo). The reference values for ascorbate alone are $K_t = 36 \pm 3$ μM and $V_{max} = 4.3 \pm 0.3$ nmol/10^6 cells. $^{*}p < 0.05$, $^{**}p < 0.001$ significant vs. [^{14}C]ascorbate alone.

be observed that the corresponding conjugate with Diclo (AA-NH-Diclo), interacts toward SVCT2 with an affinity value ($K_i = 23.5 \pm 1.8$ μM) which is two order of magnitude lower than the corresponding AA-Diclo.

Fig. 4 reports the K_t (A) and V_{max} (B) values of AA transport in the presence of different concentrations of Diclo or AA-Diclo. According to the reported values, it can be observed that Diclo is converted from a non-competitive to a competitive inhibitor upon conjugation with AA. In fact, Diclo increasing concentrations induce a great decrease in V_{max} values, whereas no difference has been registered for K_t values. On the other hand, the opposite effects have been noticed for the AA-Diclo conjugate.

Taking into account the relatively high affinity values of Diclo and AA-Diclo toward SVCT2 transporter, we have analysed their potential uptake into HRPE cells. In this regards, Figs. 5 and 6 report the HPLC chromatograms referred to Diclo and AA-Diclo, respectively, in extracellular and intracellular compartments of HRPE cells after incubation. It can be observed that Diclo is accumulated into HRPE cells (Fig. 5), whereas, its ascorbyl-prodrug AA-Diclo is not (Fig. 6).

In order to verify a potential involvement of SVCT2 transporter in the Diclo accumulation into HRPE cells, we have performed uptake experiments using drug increasing concentrations in the presence or in the absence of Na^+ (SVCT2 is a Na^+-dependent transporter) and 10 mM AA.

Table 3 reports the uptake results obtained in the different conditions. It can be observed that the Diclo accumulation into HRPE cells is concentration dependent both in the absence (NaCl is substituted by choline chloride) and in the presence of Na^+. On the other hand, the substitution of NaCl with choline chloride induces a drastic reduction of Diclo uptake, suggesting the presence of a sodium dependent transport. A comparison between data reported in the first and the last column of Table 3, indicates that Diclo transport is not affected by the presence of 10 mM AA. Taking into account that this ascorbate concentration allows to saturate the SVCT2 transporters, it is possible to conclude that the accumulation of Diclo into HRPE cells is

Table 3
Diclo uptake in HRPE cells obtained in the presence or absence of 140 mM NaCl and 10 mM ascorbate

Extracellular Diclo (μM)	140 mM NaCl	140 mM choline chloride	140 mM NaCl, 10 mM ascorbate
10	0.10 ± 0.01	0.0015 ± 0.0003	0.11 ± 0.01
30	0.18 ± 0.02	0.005 ± 0.002	0.16 ± 0.02
50	0.29 ± 0.02	0.02 ± 0.01	0.30 ± 0.02
100	0.53 ± 0.06	0.06 ± 0.02	0.57 ± 0.05
300	0.62 ± 0.07	0.08 ± 0.02	0.70 ± 0.06
500	0.78 ± 0.07	0.12 ± 0.03	0.73 ± 0.07
1000	1.25 ± 0.09	0.14 ± 0.03	1.32 ± 0.09

Data are expressed as nmol/10^6 cells/60 min.

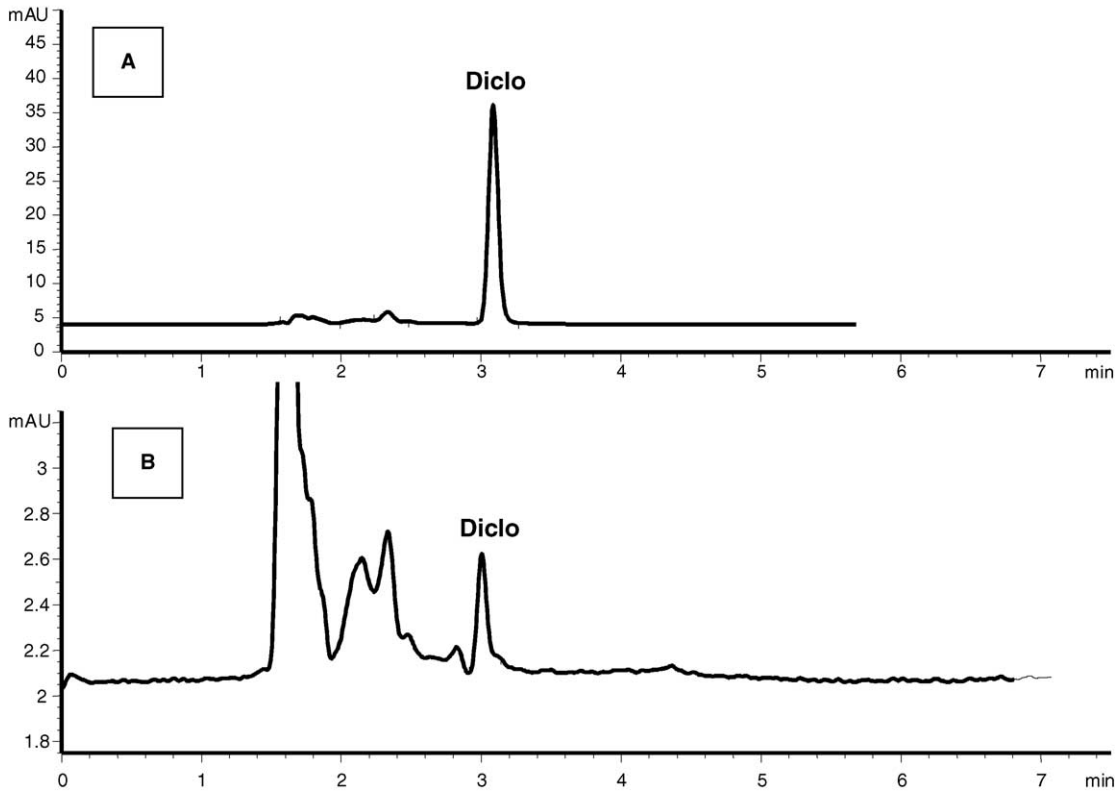


Fig. 5. HPLC chromatograms for 1 mM diclofenamic acid (Diclo) in extracellular fluid of HRPE cells (A) and for the related intracellular fluid after incubation at 37 °C for 60 min (B).

not SVCT2 mediated. These results suggest therefore the existence of a Na^+-dependent transporter for Diclo in HRPE cells, different from SVCT2.

4. Discussion

Ascorbic acid is an essential nutrient which humans need but are not able to synthesize (Nishikimi et al., 1994; Rice, 2000). The uptake and the distribution of this nutrient is therefore of fundamental importance: these functions are guaranteed by a new class of transporters of AA, named SVCT, which has been recently characterized (Friedman and Zeidel, 1999; Tsukaguchi et al., 1999). In particular, SVCT1 subtype is responsible for AA absorption from intestine and to recover it by kidneys, whereas SVCT2 subtype allows to accumulate it in the brain and eye, where it functions as antioxidant, neuromodulator for acethylcoline and noradrenaline release (Rose and Bode, 1991; Gilchrest, 1999).

A pro-drug approach, based on conjugation with AA, may be a useful tool in order to obtain potential stabilization and targeting of the drug into the brain. In fact, the pro-drug degradation may constitute a drug sustained release system which is non-toxic, being vitamin C the only co-metabolite. Moreover, as recently proposed by us, pro-drugs obtained by conjugation with AA may be recognized and transported by SVCT2 into the central nervous system (Manfredini et al., 2002). Indeed, SVCT2 is expressed by neuroepithelial cells of the choroid plexus and the retinal pigmented epithelium, allowing the AA transport in cerebral spinal fluid and aqueous humor, respectively (Friedman and Zeidel, 1999). In our in vitro studies of drug and pro-drug interactions with SVCT transporters in HRPE cells, we have demonstrated a selective expression of SVCT2 subtype (Manfredini et al., 2002).

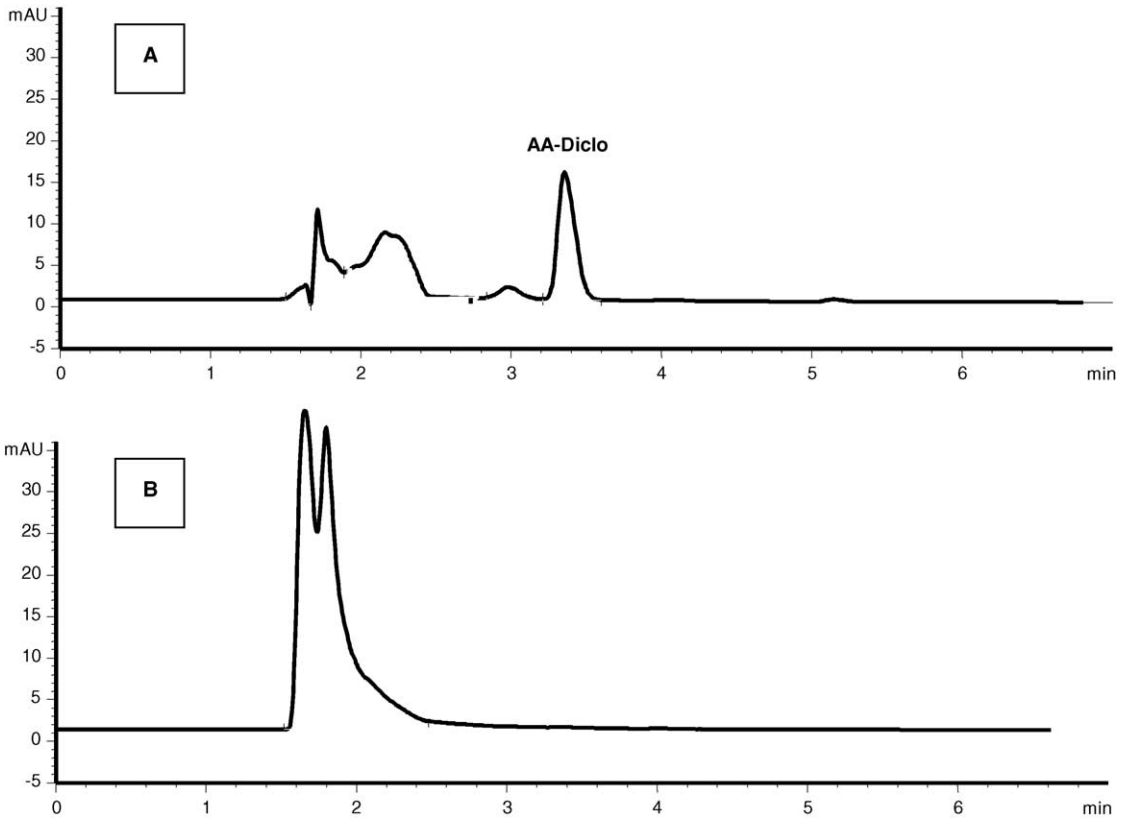


Fig. 6. HPLC chromatograms for 10 μM ascorbyl conjugate of diclofenamic acid (AA-Diclo) in extracellular fluid of HRPE cells (A) and for the related intracellular fluid after incubation at 37 °C for 60 min (B). Any uptake of AA-Diclo has been registered.

Diclo has been chosen for this type of studies because of its poor stability after systemic administration (Todd and Sorkin, 1988; Sharf et al., 1999) and its poor delivery into the brain (Zecca et al., 1991).

The AA-Diclo hydrolysis studies depicted in Fig. 2 indicate that a controlled release of Diclo can be achieved in physiological fluids. This conjugate was chosen in view of the absence of toxicity of the ascorbate metabolite. The half lives values obtained in a buffer solution (pH 7.4) and in human plasma are very similar (around 10 h, Table 1), suggesting the absence of any enzymatic hydrolysis in plasma for this prodrug. The observed degradation can be therefore attributed to the plasma pH value. On the other hand, the half-life value obtained in human blood (around 3.5 h, Table 1) indicates the presence of enzymatic activity for AA-Diclo hydrolysis which appears therefore localized in the cellular compartment. These results suggest the conjugation of Diclo with AA as a potential solution to enhance the in vivo stability of this drug.

In order to get a deeper insight into the pattern of the binding of AA-Diclo to the SVCT2 transporters we decided to explore the effects of different types of linkage between Diclo and the ascorbic acid molecule. Indeed, during a preliminary investigation (Manfredini et al., 2002), an ester bond was selected in order to consent the required reversibility of the linkage, necessary for the release from the parent drug in the brain. A different linkage could influence the conformation of the molecule, and give important information useful to draw suggestions on the structural requisites for the interaction with transporters. As a matter of fact an amide moiety represents an isosteric substitution of the ester bond and carries possible *E*/*Z* like geometric isomerism and restricted conformational degrees of freedom as

compared to the ester. Moreover, the 6-amino-ascorbic acid (AA-NH_2), also used for SVCT2 affinity studies, would be itself an interesting tool in the investigation of the structural requisite for the interaction with the transporters.

AA-NH_2 resulted totally unable to interact with SVCT2 (Fig. 3) indicating that substitution of the hydroxyl moiety in position 6 of AA for an amino group was detrimental for SVCT2 affinity. This behaviour may be probably ascribed to the fact that, under physiological conditions, the amino group can be protonated giving a zwitter ionic form that interfere with the transport mechanism. Indeed, at physiological pH ascorbic acid is present in its anionic form that probably interacts with positive centres, to form ionic bonds, within the transporters. As a matter of fact, when the amino group was acylated, the loss of basic properties probably restored the affinity for the transporters (see AA-NH-Diclo). Substitution of 6-OH with a bromine to give the 6-bromo-ascorbic acid (Manfredini et al., 2002) gave a derivative which resulted even more potent than AA. 6-Br-AA, as well as AA, are both mono anions in agreement with the above-described hypothesis of transport mechanism. In this latter case substitution with bromine, probably because of additional interaction within the site, resulted in an increase of affinity toward the transporters.

However, these data are too preliminary to advance highlight the exact mechanism at the base of the observed occurrence. Other variables are certainly involved in the interaction and transport. Indeed, our results also indicate that the AA-NH-Diclo derivative has reduced SVCT2 affinity both in the respect to the free drug and conjugate with AA (Table 2). In this case, the reduced affinity toward the corresponding AA-Diclo conjugate may be explained by the reduced conformational degree of freedom characteristic of the amide bond as compared to the ester one.

Uptake studies, conducted on the compounds characterized by the higher affinity values for SVCT2 (Table 2), indicate that the AA-Diclo conjugate, which acts as a competitive inhibitor (Fig. 4), is not transported into HRPE cells (Fig. 6). Diclo, which acts as a non-competitive inhibitor (Fig. 4), appears instead transported into the cells (Fig. 5), even if the uptake is not SVCT2 mediated (Table 3). Although the picture is far to be completed, our results have anyway allowed to support the existence in HRPE cells of a Na^+-dependent transport system for this drug, opening new perspectives in its targeting into the brain.

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