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Evidence for (+)-cicletanine sulfate as an active natriuretic metabolite of cicletanine in the rat

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Abstract

It was previously shown that the urinary sulfo-conjugate metabolite of cicletanine (cicletanine sulfate), and not free cicletanine, is salidiuretic in rats. Here we investigated potential differences between the resolved (±) enantiomers of cicletanine sulfate. Two groups of rats ($n = 10$) received either (+)- or (−)-cicletanine p.o. High performance capillary electrophoresis revealed that the 24-h urinary excretion of (+)-cicletanine sulfate was 5 times higher than that of (−)-cicletanine sulfate (18.9% vs. 3.8% of the oral dose). The same relative trend was observed after 5 and 10 days of oral administration. Following direct administration into the renal artery of anesthetized rats, (+)-cicletanine sulfate was 3–4 times more potent, in terms of active doses, than (−)-cicletanine sulfate to increase sodium excretion ($ED_{50} = 1.86 \pm 0.28$ mg/kg vs. 6.1 ± 1.0 mg/kg, mean ± S.E.M., $n = 4$). The maximal natriuretic potency of (+)-cicletanine sulfate was intermediate between that of furosemide and DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonate). In rat erythrocytes, (+)-cicletanine sulfate was between 2 and 3 times more potent to inhibit the Na^+-dependent Cl^-/HCO_3^- anion exchanger than (−)-cicletanine sulfate ($IC_{50} = 61 \pm 3$ μM vs. 142 ± 31 μM, $n = 4$). In conclusion, (+)-cicletanine was more sulfo-conjugated and a more potent natriuretic agent in rats than (−)-cicletanine. These results strongly suggest that (+)-cicletanine sulfate is the active natriuretic metabolite of racemic cicletanine in rats. This compound may probably act by inhibiting the Na^+-dependent Cl^-/HCO_3^- anion exchanger at the cortical diluting segment.

Keywords: Cicletanine; Sulfate; Kidney; Cell membrane; Ion transport; Natriuresis; Diuresis; Na^+ transport; Capillary electrophoresis

1. Introduction

Cicletanine is a new antihypertensive compound which exerts a direct action at the vascular wall, and at high doses induces significant natriuresis (Hadj-Aissa et al., 1987; Malherbe et al., 1988; Auguet et al., 1988; Bouthier et al., 1988; Ruchoux et al., 1989; Silver et al., 1990; Ebeigbe and Cabanié, 1991; see also structure in Fig. 1). This natriuretic action of cicletanine seems to be exerted at the cortical thick ascending limb of Henle (Hadj-Aissa et al., 1987).

Pharmacokinetic and metabolic studies have shown that cicletanine is rapidly and almost fully metabolized into sulfo- and glucuro-conjugated derivatives (Fredj, 1988; Pruñonosa et al., 1992a; Fig. 1). In healthy volunteers, both metabolic conjugation pathways, glucuronidation and sulfation, occur to a similar extent (Pruñonosa et al., 1992a). The conjugated metabolites undergo an enterohepatic cycle and are subsequently excreted by the kidney (Fredj, 1988; Pruñonosa et al., 1992a).

We recently reported that the urinary sulfo-conjugate metabolite (cicletanine sulfate), and not free cicletanine, is salidiuretic in rats (Garay et al., 1992; for salidiuretic actions of urinary sulfate metabolites see Garay et al., 1990, 1991; Shinkawa et al., 1992). Moreover, we found that cicletanine sulfate was able to inhibit the erythrocyte Na^+-dependent Cl^-/HCO_3^- an-

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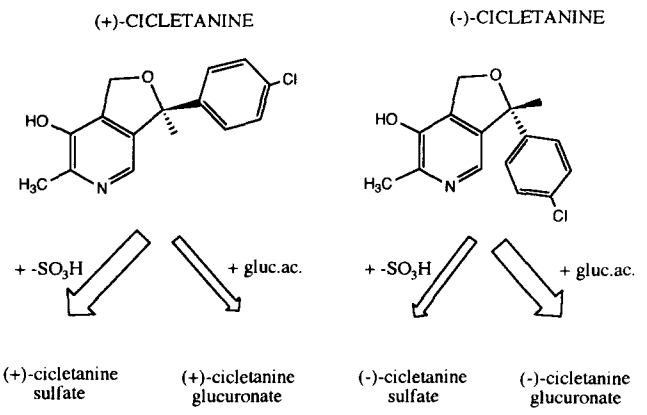

Fig. 1. Structures and metabolism of (−) and (+) enantiomers of cicletanine. Evidence is provided here for (+)-cicletanine sulfate being the active salidiuretic metabolite of cicletanine.

ion exchanger (Garay et al., 1992). A similar Na^+-dependent Cl^-/HCO_3^- exchanger seems to be *luminally* located in the cortical diluting segment (Friedman and Andreoli, 1982; Good, 1985), further suggesting that cicletanine acts at this segment level (Hadj-Aissa et al., 1987).

Fig. 1 shows that cicletanine possesses an asymmetric carbon and therefore it has two enantiomers. Recently, Buchholz et al. (1992) investigated the natriuretic and diuretic effects of orally administered cicletanine and its resolved enantiomers in conscious, hydrated, normotensive rats. These authors reported that the (+) enantiomer produced salidiuresis qualitatively similar to that of racemic cicletanine. Conversely, (−)-cicletanine was only slightly natriuretic and this at high doses (Buchholz et al., 1992).

The above results suggested to us that (+)-cicletanine sulfate was perhaps the active salidiuretic metabolite of cicletanine. Therefore, the urinary excretion profile of sulfo- and glucuro-conjugated metabolites was investigated after oral administration of either (+)- or (−)-cicletanine to rats. Moreover, the resolved ((+) and (−)) enantiomers of cicletanine sulfate were investigated for salidiuretic activity by direct administration into the renal artery of anesthetized rats, and for inhibitory activity on membrane ion transport.

2. Materials and methods

2.1. Pharmacokinetic and metabolic studies

Male Wistar rats weighing 250–270 g (DEPRE, Saint Doulchard, France) were housed in individual metabolic cages devised to prevent faeces-urine contact. All animals were maintained in a humidity- and temperature-controlled room and were fed on a standard diet (U.A.R., Villemoisson, France). Tap water was given ad libitum.

After 3 days of adaptation to the metabolic cages, two groups of 10 rats were given either (+)- or (−)-cicletanine p.o. at a dose of 10 mg/kg (in a volume of 1 ml/1 mg of 5% syrup). Urine was collected daily for 10 days and was kept at −70°C.

The urinary excretion profile of free cicletanine and conjugated metabolites was investigated by using a previously described high performance capillary electrophoresis method (Pruñonosa et al., 1992b).

2.2. Salidiuresis in rats

The resolved ((+) and (−)) enantiomers of cicletanine sulfate were both tested for salidiuretic activity by renal intra-arterial administration in rats according to a previously published protocol (Garay et al., 1992). Briefly, male Wistar rats (250–300 g) were anesthetized with 60 mg/kg of pentobarbital intraperitoneally, and then loaded with 20 ml/kg of 0.9% NaCl and tracheotomized. Following midline abdominal incision, the left ureter was catheterized and the effluent collected in an Eppendorf tube. The upper mesenteric artery was catheterized and the tip placed in the aorta near the left renal artery. After 1 h of stabilization, 2 basal clearance periods of 20 min were allowed.

To investigate the effect of drugs, the compounds were diluted in physiological saline and different doses were given to different animals. Following the intra-arterial administration of compounds, 3 further clearance periods of 20 min were allowed.

2.3. Measurement of Na^+-dependent Cl^-/HCO_3^- anion exchanger in rat erythrocytes

Na^+-dependent Cl^-/HCO_3^- anion exchanger was measured in rat erythrocytes by using a method previously described for human red blood cells (Garay et al., 1992). Briefly, DIDS (4,4′-diisothiocyano-2,2′-disulfonic stilbene)-sensitive Li^+ efflux from $LiCO_3^-$-loaded erythrocytes was taken as a marker of the Na^+-dependent Cl^-/HCO_3^- anion exchanger (Garay et al., 1992).

2.4. Ion fluxes in human red blood cells

Ion fluxes mediated by 4 discrete membrane ion carriers in human erythrocytes were measured by using previously published protocols (Garay et al., 1984, 1990, 1991, 1992). DIDS-sensitive Li^+ efflux from $LiCO_3^-$-loaded red cells was taken as a marker of the Na^+-dependent Cl^-/HCO_3^- anion exchanger (Garay et al., 1992). Bumetanide-sensitive Na^+ efflux in Mg-sucrose medium was taken as a measure of outward Na^+-K^+-Cl^- co-transport (Garay et al., 1990, 1991). DIOA (*R*(+)-[(2-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-

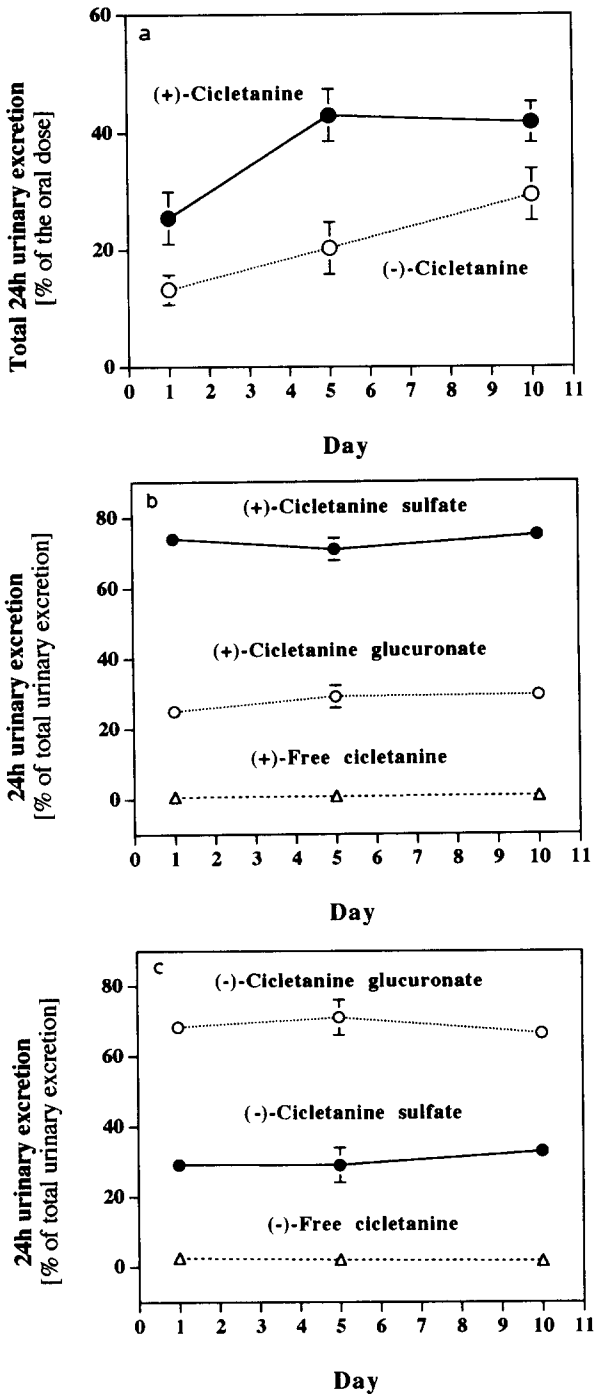

Fig. 2. (a) Total 24-h urinary excretion of mother compound and conjugated metabolites in rats orally treated for 10 days with 10 mg/kg/day of either (+)- or (−)-cicletanine. It can be seen that the total urinary excretion of (+)-cicletanine was higher than that of (−)-cicletanine. (b) Percent distribution of the total urinary excretion of (+)-cicletanine into free cicletanine and conjugated metabolites. It can be seen that most (+)-cicletanine was excreted in the form of sulfoconjugated metabolite, less as glucuronide metabolite, with very small amounts of free (+)-cicletanine. (c) Percent distribution of the total urinary excretion of (−)-cicletanine into free cicletanine and conjugated metabolites. It can be seen that most (−)-cicletanine was excreted in the form of glucuronide metabolite, less as sulfoconjugated metabolite, with very small amounts of free (−)-cicletanine.

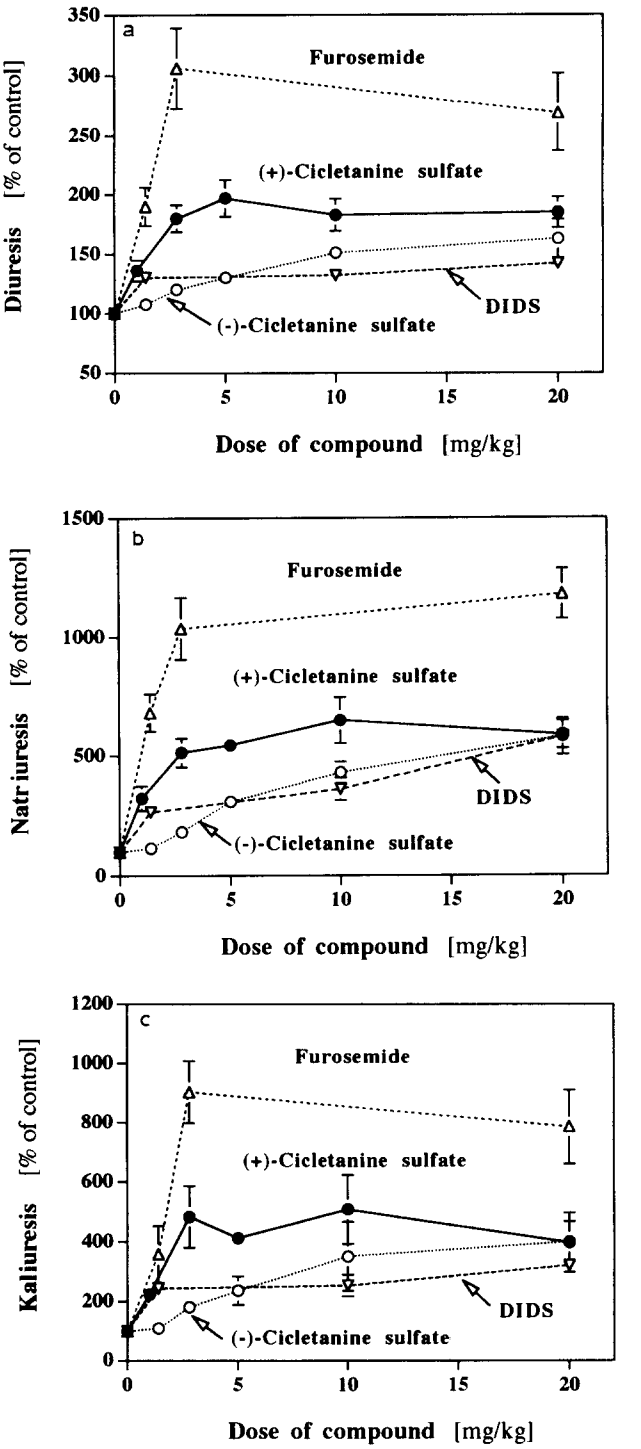

Fig. 3. (a) Concentration-response curves for the diuretic activity of renal intra-arterial (+)- and (−)-cicletanine sulfate in rats. Values represent means ± S.E.M. (n = 4). Basal values were 300–500 μl/20 min. (b) Natriuretic activity of renal intra-arterial (+)- and (−)-cicletanine sulfate (mean ± S.E.M., n = 4). Basal values were 25–45 μmol/20 min. In terms of active doses (+)-cicletanine sulfate was more potent than the (−) enantiomer. Moreover, its maximal natriuretic effect was close to that of DIDS (n = 3), and about half that of furosemide (n = 3). (c) Kaliuretic activity of renal intra-arterial (+)- and (−)-cicletanine sulfate (mean ± S.E.M., n = 4). Basal values were 25–45 μmol/20 min. Both enantiomers showed a similar profile on kaliuresis than in natriuresis and diuresis.

1-oxo-1*H*-inden-7-yl)oxy]acetic acid)-sensitive K^+ efflux in fresh cells incubated in hypotonic media was taken as a measure of K^+-Cl^- co-transport (Garay et al., 1990, 1991, 1992). Ouabain-sensitive Na^+ efflux in K-Mg-sucrose medium was equated to Na^+, K^+ pump activity (Senn et al., 1988).

2.5. Drugs

The (−) and (+) enantiomers of cicletanine and cicletanine sulfate were specifically synthesized for this study by André Esanu (I.H.B., Le Plessis-Robinson, France). DIOA was a gift from Edward J. Cragoe, Jr. (Nacogdoches, Texas, USA). Bumetanide was obtained from Leo Laboratories (Vernouillet, France). DIDS and all other chemicals were either from Merck or Sigma (distributed through Coger, Paris, France).

To study the effect on membrane ion transport, the compounds were added from freshly prepared, concentrated stock solutions in water or DMSO (dimethyl sulfoxide), provided that the final DMSO concentrations had no effect per se on ion transport. All drugs were evaluated in a manner so as to generate concentration-response curves.

2.6. Statistical analysis

Values are given as means ± S.E.M. Differences in means were tested by using the unpaired Student's *t*-test.

3. Results

3.1. Pharmacokinetic and metabolic properties of (+)- and (−)-cicletanine p.o.

The rats were treated p.o. with either (+)- or (−)-cicletanine and followed for 10 days as described in Materials and methods. Fig. 2a shows the *total* (free compound + conjugated metabolites) 24-h urinary excretion of each cicletanine enantiomer. It can be seen that, in the first 24 h, total urinary excretion of (+)-cicletanine (25.5 ± 4.5% of the oral dose, mean ± S.E.M., $n = 10$) was about twice as much as that of (−)-cicletanine (13.2 ± 2.5%). The percent recovery of each enantiomer in urine increased at days 5 and 10, and their relative differences tended to be partially reduced (Fig. 2a).

Fig. 2b shows the percent distribution of total urinary (+)-cicletanine in free cicletanine and conjugated metabolites. It can be seen that most (+)-cicletanine (70–75%) was excreted in the form of sulfoconjugated metabolite. The remaining 25–30% was excreted as glucuronide metabolite, with very small amounts of free (+)-cicletanine (0.5–1%, Fig. 2b). This pattern was maintained constant throughout the 10 days of observation.

Fig. 2c shows that most (−)-cicletanine (65–70%) was excreted in the form of glucuronide metabolite. The remaining 30–35% was excreted as sulfoconjugated metabolite, with very small amounts of free (−)-cicletanine (≈ 2%, Fig. 2c). This pattern was maintained constant throughout the 10 days of observation.

3.2. Salidiuretic activity of renal intra-arterial (+)- and (−)-cicletanine sulfate

The resolved (+) and (−) enantiomers of cicletanine sulfate were tested for salidiuretic activity by direct administration into the rat renal artery. Fig. 3a, b and c shows concentration-response curves for both compounds on diuresis, natriuresis and kaliuresis respectively (basal values are shown in the figure legends). It can be seen that (+)-cicletanine sulfate induced : (i) a 6-fold increase in natriuresis with ED_{50} = 1.86 ± 0.28 mg/kg (Fig. 3b), (ii) a similar increase in kaliuresis (Fig. 3c), and (iii) a less potent diuretic action (Fig. 3a). Conversely, (−)-cicletanine sulfate was a much less potent salidiuretic compound (Fig. 3a, b and c). Indeed, doses higher than 2 mg/kg were required to induce significant natriuresis (Fig. 3b), with a maximal effect at 20 mg/kg and $ED_{50} = 6.1 \pm 1.0$ mg/kg ($P < 0.05$ for the ED_{50} between both enantiomers).

The salidiuretic action of cicletanine sulfate enantiomers was compared with that of reference compounds. Fig. 3b shows that both enantiomers induced a maximal natriuresis (587 ± 58% and 580 ± 40% of baseline values for (+)- and (−)-cicletanine sulfate respectively) similar to that obtained with DIDS (578 ± 77%), but only half that induced by furosemide (1181 ± 105%). Moreover, the ED_{50} for both enantiomers was between that of furosemide (1.51 ± 0.53 mg/kg) and DIDS (8.9 ± 2.9 mg/kg). Similar results were obtained for the diuretic and kaliuretic actions (Fig. 3a and c).

3.3. Membrane ion transport systems

We previously reported that racemic cicletanine sulfate inhibits the erythrocyte Na^+-dependent Cl^-/HCO_3^- anion exchanger with a potency intermediate between that of furosemide and DIDS (see Garay et al., 1992). Fig. 4 shows the effect of the resolved enantiomers on this transport system in rat erythrocytes. It can be seen that (+)-cicletanine sulfate exhibited an inhibitory potency ($IC_{50} = 61 \pm 3$ μM) about twice that of (−)-cicletanine sulfate ($IC_{50} = 142 \pm 31$ μM).

The compounds were re-examined on human erythrocytes. Regarding the Na^+-dependent Cl^-/HCO_3^-

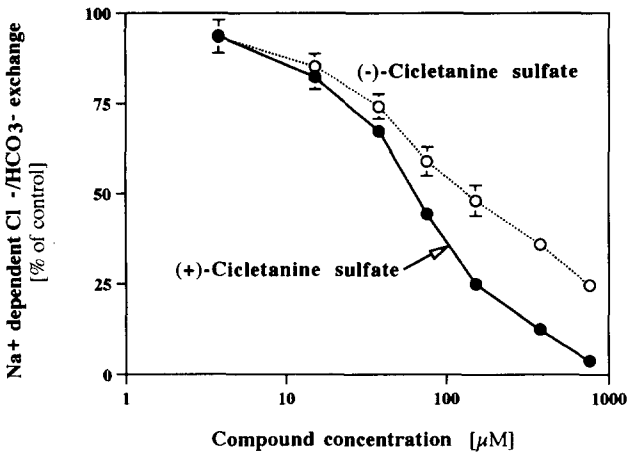

Fig. 4. Inhibition by (+)- and (−)-cicletanine sulfate of Na^+-dependent Cl^-/HCO_3^- exchange in rat erythrocytes. Values are given as means ± S.E.M. of 4 experiments. It can be seen that (+)-cicletanine sulfate exhibited an inhibitory potency ($IC_{50} = 61 \pm 3$ μM) about twice that of (−)-cicletanine sulfate ($IC_{50} = 142 \pm 31$ μM).

anion exchanger, the obtained IC_{50} values (66 ± 28 μM and 130 ± 30 μM for (+)- and (−)-cicletanine sulfate respectively, $n = 3$) were very similar to those found in rat erythrocytes. Moreover, similarly to racemic cicletanine sulfate, the (+) and (−) isomers were modest inhibitors of the Na^+-K^+-Cl^- co-transport system ($IC_{50} = 986 \pm 292$ μM and 1062 ± 418 μM for (+)- and (−)-cicletanine sulfate respectively, $n = 3$). Moreover they were poor inhibitors of the K^+-Cl^- co-transport system and were unable to modify Na^+,K^+ pump activity (data not shown).

4. Discussion

Buchholz et al. (1992) have found that the salidiuretic activity of racemic cicletanine (given orally to rats) is mostly due to its (+) enantiomer. Moreover, we have previously reported that racemic cicletanine acts via its sulfoconjugated urinary metabolite: cicletanine sulfate (Garay et al., 1992). We supposed that the *link* between both observations was that urinary (+)-cicletanine sulfate was the main metabolite responsible for the salidiuretic action of orally given racemic cicletanine. The present investigation supported this working hypothesis.

Several observations suggested that (+)-cicletanine sulfate was the active salidiuretic metabolite of orally given racemic cicletanine in rats. First, the urinary excretion of (+)-cicletanine sulfate was considerably higher than that of (−)-cicletanine sulfate. Thus, data in Fig. 2a, b and c allowed us to calculate that, following a single oral administration of each cicletanine enantiomer, the 24-h urinary excretion of (+)-cicletanine sulfate was 5 times higher than that of (−)-cicletanine sulfate (18.9% vs. 3.8% of the oral dose). Moreover, the urinary excretion of (−)-cicletanine glucuronate was 40% higher than that of (+)-cicletanine glucuronate (9.0% vs. 6.4% of the oral dose). These results were in line with a preliminary study of Pruñonosa et al. (1992b) suggesting *partial stereoselectivity* for the sulfo- and glucuroconjugation of cicletanine. These authors have recently developed a high performance capillary electrophoresis procedure for the determination of (+)- and (−)-cicletanine enantiomers in human plasma and urine (Pruñonosa et al., 1992b). Preliminary data for normal subjects were in line with our data for rats, i.e., (−)-cicletanine was more glucuroconjugated than the (+) enantiomer (Pruñonosa et al., 1992b; see also Fig. 1).

Further evidence for (+)-cicletanine sulfate as the active salidiuretic metabolite of cicletanine was provided by experiments testing the salidiuretic activity of compounds after direct administration into the rat renal artery. In these experiments, (+)-cicletanine sulfate was 3–4 times more potent to induce salidiuresis than the (−) enantiomer. Maximal natriuresis with (+)-cicletanine sulfate was similar to that obtained with DIDS and lower (about half) than that of furosemide. It is important to mention that relatively high doses of DIDS were required to obtain maximal natriuresis (Fig. 3b). This can be perhaps due to the fact that DIDS is a *di*-sulfonic acid, a property which may limit its excretion into the proximal tubule (Ulrich and Rumrich, 1988).

Final evidence for (+)-cicletanine sulfate as the active salidiuretic metabolite of cicletanine was provided by measurement of membrane ion fluxes. Thus, (+)-cicletanine sulfate was considerably more potent to inhibit the DIDS-sensitive Na^+-dependent Cl^-/HCO_3^- exchanger than the (−) enantiomer. Diuretic drugs act on the luminal side of tubular cells, and a *luminally* located Na^+-dependent Cl^-/HCO_3^- exchanger has been reported in the cortical diluting segment of the mouse (Friedman and Andreoli, 1982) and rat (Good, 1985) kidney. Moreover, Hadj-Aissa et al. (1987) have found that cicletanine (orally given to humans) acts at the cortical diluting segment. In the rat, (+)-cicletanine induces natriuresis at concentrations higher than 1–3 mg/kg p.o. (Buchholz et al., 1992), i.e., at excreted concentrations of (+)-cicletanine sulfate (> 15–45 μM; calculated from data in Fig. 2a and b) which inhibit the Na^+-dependent Cl^-/HCO_3^- exchanger (Fig. 4). In healthy human volunteers after administration of a 50 mg single oral dose, the excreted concentrations of cicletanine sulfate were ≈ 50–90 μM (during the first 6 h, calculated from data in Pruñonosa et al. (1992a); for a discussion of the relation between plasma cicletanine concentrations and vasorelaxant doses see Silver and Cumiskey (1991)). Taken together, all these results suggest that (+)-cicletanine sulfate

may act at the Na^+-dependent Cl^-/HCO_3^- exchanger of the cortical diluting segment. However, we cannot exclude other possibilities, such a natriuretic action via the inhibition of cGMP phosphodiesterase (Silver et al., 1990).

It is important to mention that Buchholz et al. (1992) have found that orally given (+)-cicletanine was 1–2 orders of magnitude more potent a salidiuretic compound than the (−) enantiomer. Our results suggest that this relatively large range can result from both: (i) greater sulfoconjugation of (+)-cicletanine and (ii) higher salidiuretic potency of (+)-cicletanine sulfate. Our values for the relative (+)/(−) enantiomer ratios (5-fold higher urinary sulfate excretion and 3–4 times higher salidiuretic potency) predict a 15–20 times higher natriuretic potency (in terms of active doses) for orally given (+)-cicletanine in rats, a value in line with the observation of Buchholz et al. (1992).

In conclusion, the data strongly suggest that (+)-cicletanine sulfate is the active salidiuretic metabolite of orally given racemic cicletanine in rats. This compound probably acts by inhibiting the apical Na^+-dependent Cl^-/HCO_3^- anion exchanger in the cortical diluting segment.

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