Torasemide Transport by Organic Anion Transporters Contributes to Hyperuricemia

Yohannes Hagos,* Andrew Bahn,* Stefan V. Vormfelde,† Jürgen Brockmüller,† and Gerhard Burckhardt*

*Abteilung Vegetative Physiologie und Pathophysiologie and †Abteilung Klinische Pharmakologie Klinikum, Georg August Universität, Göttingen, Germany

ABSTRACT

The high renal clearance of torasemide, the most potent loop diuretic, suggests active tubular secretion in the proximal tubule. Previous studies implicated the organic anion transporters (OAT) in this process; human OAT1 (hOAT1) and hOAT3 are found on the basolateral surface of proximal tubule cells, and hOAT4 is found on the luminal surface. This study sought to determine the mechanism underlying renal elimination of torasemide and to elucidate the drug’s effect on renal urate secretion, because hyperuricemia is a concerning adverse effect. Torasemide and its metabolites were transported into stably transfected HEK293 cells by hOAT1, hOAT3, and hOAT4 and out of the cells by hOAT3 and hOAT4. These data suggest that basolateral hOAT3 and luminal hOAT4 are likely responsible for the translocation of torasemide across the proximal tubule cell. Regarding urate handling, torasemide and its metabolites did not interact with human URAT1, but competitive inhibition of the basolateral OAT for urate may reduce tubular secretion. Furthermore, because hOAT4 can reabsorb urate from the urinary lumen, increased urate reabsorption may occur as exchange for the secretion of torasemide and its metabolites. In support of this hypothesis, fractional excretion of urate was reduced in 95 healthy volunteers after torasemide administration. In summary, this study determined the affinity of OAT for torasemide and its metabolites and proposed a mechanism underlying torasemide-induced hyperuricemia that does not involve the human URAT1-mediated transport affected by other loop diuretics.


Torasemide (1-isopropyl 3-[4-(3-methylphenylamino)-3-pyridinesulphonyl]urea) is the most active member of a newer generation of loop diuretics. It is used for the treatment of both acute and chronic congestive heart failure and hypertension. Oral torasemide dosages without diuretic effect (2.5 to 5 mg/d) have been used to treat essential hypertension, resulting in a decreased diastolic BP to <90 mmHg within 60 to 80 d. Torasemide is so far the only loop diuretic that has been reported to reduce high BP effectively at the low dosage of 2.5 mg/d.1 After oral administration, the maximal plasma concentration of torasemide was reached after 1 h, showing a bioavailability of >80% in healthy individuals. In contrast, the classical loop diuretic furosemide has a bioavailability of only 26 to 60%.2,3 Torasemide is highly bound to plasma proteins, and its elimination half-life is almost 6 h, which is approximately three-fold higher than that of furosemide or bumetanide. Torasemide shares a similar mechanism of inhibition of Na⁺-K⁺-2Cl⁻ reabsorption at the thick ascending limb of Henle’s loop with furosemide or bumetanide. Studies on urinary dose-response curves documented torasemide to be five-fold as potent as furosemide.4

Diuretics undergo different hepatic metabolism.
Bumetanide and torasemide are metabolized by the cytochrome P450 pathway, whereas furosemide is glucurononidated. Only 20% of furosemide is metabolized in the liver, but almost 75% of torasemide is heptatically metabolized to M1, M3, and M5. Figure 1 illustrates schematically the hepatic metabolism pathway of torasemide. The diuretic potencies of M1 and M3 are different.\textsuperscript{3,5,6} M1 exhibits only 10\% of the diuretic effect, whereas M3 possesses a similar potency as the parent drug; M5 is diuretically inactive. A quantification of torasemide and its metabolites in urine revealed 21 to 25\% torasemide, 11\% M1, 3\% M3, and 34 to 44\% M5, resulting in a total renal clearance of >80\% for torasemide and its metabolites.\textsuperscript{2,6}

The calculated renal clearance of free torasemide (not bound to plasma protein) was approximately 640 ml/min,\textsuperscript{3} which is equal to renal plasma flow. The high clearance for torasemide indicated the involvement of active secretory mechanisms in proximal tubule cells. Simultaneous administration of torasemide and probenecid lowered the diuretic effect and reduced torasemide clearance, suggesting organic anion transporters (OAT) to be involved in renal secretion, because probenecid is the classical inhibitor of OAT.\textsuperscript{3,4} The aim of this study was to elucidate the interaction of torasemide and its metabolites with human OAT and to understand the background of the pronounced clinical advantage of torasemide on the molecular level. Furthermore, we aimed to identify the influence of torasemide on the OAT-mediated urine transport.

Human OAT (hOAT) are well-characterized transporters that belong to the solute carrier family (SLC22A; for review, see Wright and Dantzler\textsuperscript{7} and Sweet\textsuperscript{8}). They are responsible for the uptake and excretion of several endogenous and exogenous substances. Endogenous substrates of OAT are the citrate cycle metabolites, the steroid hormone derivatives estrone sulfate (ES), dehydroepiandrosterone sulfate, and cortisol; the second messenger cAMP or cGMP; and hormones such as prostaglandin E\textsubscript{2}. Exogenous compounds known to be transported by OAT are \beta-lactam antibiotics, antiviral drugs, nonsteroidal anti-inflammatory drugs, cytostatics, and diuretics.\textsuperscript{9,10} OAT1 and OAT3 are expressed on the basolateral side of proximal tubule cells, facilitating the uptake of organic anions into the cell and consequently the first step of renal secretion of these compounds. hOAT4 and human URAT1 (hURAT1) are expressed at the luminal side of proximal tubule cells. hOAT4 mediates the secretion of organic anions into the urine in exchange for chloride as well as the uptake of organic anions into the proximal tubule cells, leading to the reabsorption of ES, dehydroepiandrosterone sulfate, and urate from primary urine in exchange for \alpha-ketoglutarate.\textsuperscript{11} Human URAT1 (hURAT1) is known as the luminal urate/lactate exchanger, which plays an essential role in urate homeostasis. Mutations in hURAT1 gene, resulting in a lower renal reabsorption of urate, cause hypouricemia (lower urate plasma level).\textsuperscript{12}

Hyperuricemia may result from a high-purine diet, tumor lysis, or application of diuretics.\textsuperscript{13,14} The molecular mechanism of diuretic-induced hyperuricemia is poorly understood. hURAT1-mediated urate transport was substantially inhibited by furosemide and bumetanide, but whether it is also involved in diuretic-induced hyperuricemia is unclear.\textsuperscript{12} Reyes \textit{et al.}\textsuperscript{14} documented the involvement of torasemide in the elevation of urate plasma level, but again the molecular background of torasemide-induced hyperuricemia is unknown. Recently, we demonstrated that hOAT4 is a urate transporter, facilitating export of the diuretic hydrochlorothiazide in exchange for urate.\textsuperscript{11} Thus, hOAT4 is a candidate for causing hyperuricemia.

The interaction of OAT with diuretics has been documented,\textsuperscript{15} but torasemide and its metabolites were not tested. To determine the transport proteins involved in renal secretion of torasemide and its metabolites and consequently to understand the higher efficiency of torasemide in comparison with furosemide or bumetanide, we investigated the interaction of torasemide and its main human metabolites M1, M3, and M5 with hOAT1, hOAT3, hOAT4, and hURAT1. In addition, we evaluated the influence of torasemide on renal urate excretion in patients and provide an explanation for the interaction of torasemide with renal urate transport and for a possible torasemide-induced hyperuricemia.

\section*{Results}

\textit{cis-Inhibition of hOAT1-, hOAT3-, hOAT4-, or hURAT1-Mediated p-aminohippurate, ES, or Urate Uptake by Torasemide, M1, M3, and M5.} Initially, we tested the interaction of torasemide and its metabolites with hOAT1, hOAT3, hOAT4, and hURAT1 in \textit{cis}-inhibition studies. hOAT1-expressing HEK293 cells showed a [$^{3}$H]p-aminohippurate (PAH) uptake of 10.5 ± 0.5 pmol/10\textsuperscript{6} cells per 5 min ($n = 3$), compared with mock cells with a transport rate of 1.3 ± 0.1 pmol/10\textsuperscript{6} cells per 5 min ($n = 3$). In three separate experiments with quadruplicate assays under each condition, hOAT1-mediated uptake of [$^{3}$H]PAH was inhibited by addition of 200 \textmu{}M torasemide, M1, M3, or M5 into
the incubation buffer to 67.3 ± 3.8, 58.1 ± 6.4, 62.3 ± 4.5, or 63.1 ± 8.1%, respectively (Figure 2A).

The [3H]ES uptake measured in hOAT3- or hOAT4-expressing HEK293 cells revealed transport rates of 29.2 ± 2.7 fmol/10⁶ cells per 5 min (n = 3) and 24.7 ± 1.6 fmol/10⁶ cells per 5 min (n = 3), respectively, in comparison with 5.4 ± 0.5 fmol/10⁶ cells per 5 min (n = 3) for mock cells. hOAT3-mediated ES transport was inhibited by torasemide, M1, M3, and M5 by 67 ± 4.8, 51.2 ± 6.9, 51.4 ± 6.8, and 74.7 ± 6.8%, respectively (Figure 2B). hOAT4 transport activity was reduced with torasemide by 74.4 ± 4.2%, M1 by 44.5 ± 93%, and M3 by 68.5 ± 5.0%. M5 did not influence the transport activity of hOAT4, suggesting no interaction of hOAT4 with this metabolite (Figure 2C). The inhibition profile of hURAT1-mediated urate uptake into Xenopus laevis oocytes for torasemide and its metabolites exhibited only a slight but NS interaction with these compounds. The classical inhibitors benz bromarone and probenecid revealed a strong inhibition (Figure 2D). These data show a substantial interaction of torasemide and its metabolites with hOAT1, hOAT3, and hOAT4 but not with hURAT1.

To verify the type of inhibition (competitive or uncompetitive) and to determine the Ki values, we conducted Dixon plot analyses. Torasemide, M1, M3, and M5 revealed a competitive inhibition of PAH uptake by hOAT1 (Figure 3). Similar competitive inhibitions were obtained for ES uptake by hOAT3 and hOAT4 (data not shown), but the calculated mean Ki values are summarized in Table 1.

**trans-Stimulation of hOAT1, hOAT3, hOAT4, or hURAT1 by Preloading/Injecting Cells/Oocytes with Torasemide or Its Metabolites**

For a further understanding of the interaction of OAT and hURAT1 with torasemide, M1, M3, and M5, we performed

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**Figure 2.** cis-Inhibition of hOAT- or hURAT1-mediated PAH, ES, or urate uptake. The inhibition study was performed using 200 μM torasemide or metabolites (M1, M3, or M5) or probenecid (Probe) and 100 μM benz bromarone (Benzb). Inhibition profiles of hOAT1-mediated 1.2 μM [3H]PAH uptake (A), 20 nM [3H]ES uptake by hOAT3 (B) or by hOAT4 (C), and 100 μM [14C]urate uptake by hURAT1 (D) are documented. □, Mock cells or water-injected oocytes; △, hOAT1-, hOAT3-, or hOAT4-expressing HEK293 cells or hURAT1-expressing oocytes. All experiments were standardized by setting the control (without inhibitor “Ringer”) of each experiment to 100%. Data are means ± SEM of three independent experiments with three to four repeats each or 11 oocytes each.
trans-stimulation studies by preloading the stably transfected cells for 2 h or by injecting X. laevis oocytes with 1 mM to-
rasemide as well as its metabolites. Subsequently, uptake of labeled PAH, ES, or urate was tested.

The results of the trans-stimulation studies are presented in Figure 4. None of the preloaded substances resulted in a trans-stimulation of hOAT1-mediated PAH (Figure 4A) or hURAT1-mediated urate (Figure 4D) uptake. Actually, we observed a slight but significant trans-inhibition of PAH or urate uptake with torasemide, M1, and M5. M3 showed no difference to unloaded control cells or oocytes. Nicotinate showed, as expected, a substantial trans-stimulation of hURAT1-mediated urate uptake (Figure 4D). In contrast, hOAT3-mediated ES transport was significantly trans-stimulated by torasemide, M3, and M5 up to 154.6 ± 14.7, 146.1 ± 13.0, and 136.6 ±
11.5%, respectively (Figure 4B). The highest trans-stimulatory effect was observed for M1, which showed 2.5-fold higher ES uptake in comparison with control cells that were not pre-loaded (100 ± 4.6%). The transport activity of hOAT4 was trans-stimulated by torasemide to 147 ± 4.6%, M1 to 227 ± 5.3%, M3 to 133 ± 0.8%, and M5 to 109 ± 0.9% (Figure 4C). The profiles of trans-stimulation of hOAT3 and hOAT4 are similar and prove that torasemide and its metabolites are translocated by these two transporters. hOAT1 and hURAT1 do not seem to interact with intracellularly applied torasemide or its metabolites. It is well documented that hOAT3 and hOAT4 facilitate the transport of urate and play an important role in urate homeostasis; therefore, we examined the effect of torasemide on urate excretion in 95 healthy male subjects who were treated with 10 mg of torasemide. As a control group, 120 individuals were treated with placebo. Initially, we determined the excretion rate of torasemide in urine after administration as indicated in Figure 5A. The highest torasemide excretion occurred in the first hour (900 ± 4.6 μg/h) after treatment. Almost 2.55 ± 0.123 mg of the administered 10 mg of torasemide was excreted within 6 h in the urine. Afterwards, the excretion rate of torasemide decreased in a time-dependent manner. Torasemide amounts determined between 6 and 24 h in the urine were only 0.3 mg. The fractional excretion of urate (FEurate) within 2 h after torasemide administration was unchanged, but between 2 and 6 h, FEurate was significantly (P < 0.001) reduced by 31.7 ± 1.0%. As expected, the control group did not show any FEurate reduction after placebo administration. The time-dependent reduction of FEurate after torasemide treatment is presented in Figure 5B.

To explore the trans-stimulatory effect of torasemide and its interaction with OAT...
metabolites on urate uptake by hOAT4, we preloaded the cells with torasemide as well as the metabolites and measured [14C]urate uptake. hOAT4-facilitated urate uptake was 4.5-fold higher than in mock cells, proving that hOAT4 is a urate transporter. Torasemide and its metabolites revealed a slight but significant trans-stimulation of hOAT4-mediated urate uptake. The observed stimulatory effect was within 11 to 16% (Figure 6).

**DISCUSSION**

Torasemide is the most efficient and frequently prescribed loop diuretic. Comparative clinical and pharmacologic studies of loop diuretics documented a low total clearance of torasemide (0.6 ml/min per kg) in comparison with furosemide or bumetanide, which were 2.2 and 2.6 ml/min per kg, respectively. The duration of action of these loop diuretics was determined to be 2 to 2.5 h for furosemide, 2 h for bumetanide, and 6 h for torasemide. Our clinical study on 95 individuals who were treated with 10 mg of torasemide documents the highest torasemide excretion rate within 1 h. Almost 1 mg of torasemide was secreted within the first hour, representing 25% of the initial torasemide, and after 4 h, 2.55 mg of torasemide was excreted in the urine. The same observation was reported by Neugebauer et al.²

Urinary dose-response curves documented torasemide to be five-fold more efficient than furosemide because of its significantly longer biologic half-life and longer duration of action together with a less pronounced potassium and phosphate secretion effect; however, little is known of the background of this difference in clearance and duration of action of torasemide at the molecular level. Recent studies showed that genetic polymorphisms of CYP2C9 and hOAT4 affected the pharmacokinetics of torasemide and its clearance. So far, no correlation between torasemide clearance and genetic variations of hOAT1 or hOAT3 has been found.¹⁷,¹⁸

To elucidate the molecular mechanism of torasemide delivery into the lumen of the nephron and the renal elimination of torasemide as well as its metabolites, we examined the interaction with hOAT, namely OAT1, OAT3, and OAT4, stably transfected in HEK293 cells, and hURAT1 expressed in X. laevis oocytes. Determination of the Kᵥ values via Dixon plots revealed high affinities of hOAT1 (55 µM), hOAT3 (89 µM), and of loop diuretics documented a low total clearance of torasemide (0.6 ml/min per kg) in comparison with furosemide or bumetanide, which were 2.2 and 2.6 ml/min per kg, respectively. The duration of action of these loop diuretics was determined to be 2 to 2.5 h for furosemide, 2 h for bumetanide, and 6 h for torasemide. Our clinical study on 95 individuals who were treated with 10 mg of torasemide documents the highest torasemide excretion rate within 1 h. Almost 1 mg of torasemide was secreted within the first hour, representing 25% of the initial torasemide, and after 4 h, 2.55 mg of torasemide was excreted in the urine. The same observation was reported by Neugebauer et al.²

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and hOAT4 (47 μM) for torasemide, whereas hURAT1-mediated urate uptake was not inhibited. In all cases, the inhibition was competitive (i.e., torasemide and its metabolites bound to the same site as PAH [hOAT1] or ES [hOAT3, hOAT4]), indicating that the application of torasemide may interfere with the renal elimination of other drugs or endogenous metabolites such as urate. Torasemide showed the highest trans-stimulatory effect on hOAT3, less on hOAT4, and no such stimulation on hOAT1 or hURAT1-mediated transport.

The first comparative studies on interactions of diuretics with transport proteins were reported by Hasannejad et al.15 hOAT1 as well as hOAT3 showed higher affinity for bumetanide (hOAT1 7.6 μM; hOAT3 0.75 μM,15) and furosemide (hOAT1 18 μM; hOAT3 7.3 μM,15) than for torasemide (hOAT1 Ks 55 μM; hOAT3 Ks 89 μM, this study), consistent with a slower basolateral entry of torasemide into the proximal tubule cell. hOAT4, however, exhibited a higher affinity for torasemide (47 μM, this study) than for bumetanide (348 μM,15) and almost the same affinity for furosemide (44 μM15). hURAT1 interacted with furosemide and bumetanide, but information on the exact affinities of these substances is missing.12 In our study, hURAT1-mediated transport was not significantly altered by any of the tested compounds, suggesting that it is not involved in renal handling of torasemide.

The main metabolites of torasemide detected in human plasma or urine are M1 (−CHOH), M3 (−OH), and M5 (−COOH).19 M1 and M3 are diuretically active, but 34 to 44% of the initial dose of torasemide is detected in the urine as the inactive M5.3,6 We demonstrate that metabolites M1 and M3 interact with hOAT1, hOAT3, and hOAT4 and that M5 interacts with hOAT1 and hOAT3. In all cases, a competitive inhibition was observed with Ks values ranging between 51 and 140 μM.

Torasemide and most of its metabolites trans-stimulated ES uptake by hOAT3 and hOAT4. This finding strongly suggests that torasemide and some metabolites are translocated by hOAT3 and hOAT4. In addition, the trans-stimulation experiments provide evidence for the ability of hOAT3 to transport torasemide and its metabolites also in the absorptive direction and of hOAT4 to transport them in the secretory direction. Hence, we propose that a bidirectional transport can occur in the proximal tubule. The role of hOAT1 that was inhibited by torasemide and the metabolites but not trans-stimulated remains open. The carboxylated metabolite M5 did not show any affinity for hOAT4 in inhibition studies and exhibited nonsignificant trans-stimulation. It is, therefore, unlikely that hOAT4 is involved in the reabsorption of M5. Unlike torasemide, M1, and M3, this metabolite should undergo only secretion, which fits to the relatively high abundance of M5 in the urine.

Substantial evidence has been provided for hyperuricemia as an independent risk factor for the development of renal failure and for cardiovascular diseases.14,20 It is, therefore, interesting to understand the role of the kidneys in general and of diuretics particularly in the development of hyperuricemia. The fractional excretion of urate is 8 to 12%,13 as a result of a significant reabsorption of filtered and secreted urate in proximal tubules. The main reabsorptive urate transporter is hURAT1, and loss-of-function mutations lead to familial hyperuricemia.12 hURAT1-mediated urate reabsorption from primary urine seems not to be affected by torasemide and its metabolites, as documented by the cis-inhibition study illustrated in Figure 3D, so urate reabsorption remains untouched in comparison with furosemide or bumetanide, which inhibit hURAT1-mediated urate uptake. In contrast, torasemide and its metabolites inhibit the activity of the basolateral OAT, which means the inhibition of urate uptake from the blood, which is the first step of urate secretion. This inhibition causes elevated plasma urate level after torasemide treatment. Furthermore, we recently demonstrated that hOAT4 can act as a urate-reabsorbing transporter11; therefore, we tested whether torasemide and its metabolites may increase urate absorption through hOAT4 by trans-stimulation. Indeed, preloading of hOAT4-expressing HEK293 cells with torasemide, M1, M3, and M5 increased slightly but significantly urate uptake. Consequently, the exchange of torasemide and its metabolites against urate at hOAT4 may increase urate reabsorption and add to the mechanism described to increase the urate plasma level. This idea is further supported by our studies with 95 healthy volunteers, who were treated with 10 mg of torasemide. Here, we observed a significantly (P < 0.001) reduced fractional excretion of urate.

In summary, we determined the affinity of OAT for torasemide as well as for its metabolites. As compared with other loop diuretics, hOAT1 and hOAT3 have lower and hOAT4 higher affinities for torasemide. hURAT1 exhibits no affinity for any of these compounds, indicating no involvement of this transporter in renal excretion of torasemide or its metabolites and consequently no impact on torasemide-induced hyperuricemia. During secretion, torasemide and its metabolites may increase proximal tubular absorption of urate through hOAT4, resulting—together with an inhibition of urate secretion by hOAT1 and hOAT3—in hyperuricemia, which itself is recognized as a risk factor for renal and cardiovascular diseases.

CONCISE METHODS

Reagents and Materials

All chemicals were of analytic grade and purchased from Sigma-Aldrich (Deisenhofen, Germany) or AppliChem (Darmstadt, Germany). FBS, trypsin, and PBS were purchased from Invitrogen (Gröningen, Netherlands). Radioactive substrates such as [3H]ES, (ES, ammonium salt, 43.5 Ci/mmol), [3H]uric acid (50 to 60 mCi/mmol), and [3H]PAH (3.25 Ci/mmol) were from New England Nuclear (NEN, Boston, MA), Biotrend (Cologne, Germany), and Perkin Elmer Life Sciences (Boston, MA), respectively. Torasemide and its metabolites were a generous gift of Boehringer Mannheim (Mannheim, Germany).
Stable Transfection of hOAT1, hOAT3, and hOAT4 into HEK293 Cells

The stably transfected human epithelial kidney cell lines T-REX-HEK293-hOAT1, -3, and -4 were established by using the Flp-In expression system (Invitrogen, Gröningen, Netherlands) as described previously.²² Stably transfected HEK293 cells were selected by hygromycin (10 μg/ml) and grown in flasks in DMEM (high glucose; Invitrogen) supplemented with 10% FCS, 1% penicillin/streptomycin, and blasticidine (5 mg/L; Sigma-Aldrich, Schnelldorf, Germany). Cultures were maintained in a humidified atmosphere containing 5% CO₂ at 37°C.

Transport Measurements in HEK293 Cells

HEK293-hOAT1, -hOAT3, -hOAT4 cells and HEK293 mock cells were harvested and plated onto 24-well plates (Sarstedt, Nümbrecht, Germany) at a density of 2 × 10⁵ cells/well. Transport assays were performed after 3 d after seeding in mammalian Ringer solution (in mM: 130 NaCl, 4 KCl, 1 CaCl₂, 1 MgSO₄, 1 NaH₂PO₄, 20 HEPES, and 18 glucose (pH 7.4). The cells were washed twice with 500 μl of buffer and incubated in buffer containing 20 nM [³H]ES, 1.2 μM [³H]PAH, or 100 μM [¹⁴C]urate for 5 min, respectively. Solutions for inhibition studies included various concentrations of torasemide or its metabolites as indicated in the figures. For trans-stimulation experiments, cells were preloaded for 2 h with 1 mM torasemide or torasemide metabolites before the uptake measurements. After 5 min, the reaction was stopped by removal of the incubation buffer with the extracellular tracer and washing the monolayer three times with 500 μl of ice-cold Ringer solution. Subsequently, cells were dissolved by incubation in 0.5 ml of 1 N NaOH for 30 min and neutralized by 0.5 ml of 1 N HCl. The [³H] and [¹⁴C] contents of the cells were determined by liquid scintillation counting (Canberra-Packard).

Clinical Study

The pharmacokinetic study was conducted on 95 healthy volunteers. For avoidance of any hormone effects, the study was performed only with male individuals. Enrolled individuals were nonsmoking, white, and aged between 18 and 50 yr. Urine was collected at indicated time intervals as described previously.¹⁸ To determine the fractional excretion of urate for the control group (treated with placebo), we used urine collected from 120 male white individuals who were not treated with torasemide, as described recently.²⁴ All patients agreed on participation in the study, and the study was approved by the ethics committee of Göttingen University.

Drug Analysis

For determination of torasemide excretion in the urine, a sensitive liquid chromatography-mass spectrometry/mass spectrometry method was applied, as described previously.¹⁸

Statistical Analysis

Statistical analysis and graphic layout were performed with Microsoft Excel (Microsoft, Unterschleißheim, Germany) and SigmaPlot 2001 (SPSS Science, Chicago, IL).

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Disclosures

None.

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