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Improved solid-phase extraction and HPLC measurement of torasemide and its important metabolites

Sabine Engelhardt, Ingolf Meineke*, Jürgen Brockmöller

Department of Clinical Pharmacology, University Medical Center of the Georg August University Göttingen, Robert-Koch-Str. 40, D-37075 Göttingen, Germany

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Abstract

Torasemide is a "loop type" diuretic drug. For pharmacokinetic studies sensitive analytic methods are essential for authentic qualitative and quantitative information. A robust, selective and sensitive HPLC method is described for the simultaneous determination of torasemide, its major metabolite M5 and its active metabolites M1 and M3 and an internal standard within 17 min. Solid-phase extraction with C_2-cartridges was used for the clean-up of plasma samples. The chromatographic separation was carried out on a CN-column with a mobile phase consisting of perchloric acid (0.02 M, pH 2.5)/acetonitrile (90/10, v/v)). The calibration range used reached from 20 to 1000 ng/ml for all analytes. Coefficients of variation were less than 10% at every calibration point for each analyte. Plasma concentrations in samples obtained from volunteers in the course of a clinical study could be reliably measured with this method. Median maximum concentrations in plasma after a 10 mg oral dose during a 24 h study interval were located at 1 h for torasemide, 1 h for M1 and 2 h for M5. Concentrations between 2226 and <20 ng/ml for torasemide, between 159 and <20 ng/ml for M1 and between 420 and <20 ng/ml for M5 were observed.

Keywords: Torasemide; Loop type diuretics; HPLC; Solid-phase extraction; Pharmacokinetics

1. Introduction

Torasemide (1-isopropyl-3-[[4-(3′-methylphenylamine)pyridin]-3-sulfonyl]urea) belongs to the class of loop diuretics with a high-ceiling effect [1]. Because of its high potency low therapeutic doses are required. Torasemide is well absorbed and yields a bioavailability of about 80–90% [2]. It is highly bound to protein (99%). The volume of distribution is determined as 0.2 l/kg [2]. Torasemide undergoes extensive hepatic metabolism including hydroxylation at various positions, oxidation and reduction (Fig. 1). The most important metabolites in humans were M1, M5 and M3 with recoveries in urine of 10–12% for M1, 30–44% for M5 and about 2% for M3. Only 20–25% of the parent drug is recovered unchanged in urine [2,3].

Torasemide is secreted into the proximal tubular system by an anion-transport-mechanism. It inhibits the Na/K/2Cl-carrier in the thick ascending limb of the loop of Henle, resulting in increased saluresis and diuresis [1,3].

Sensitive analytical methods to determine torasemide and its metabolites in plasma simultaneously are required to study the pharmacokinetics and pharmacodynamics.

Few chromatographic methods were found in the literature for the determination of torasemide. These include a combined gas chromatographic-mass spectrometric method [4] as well as a few liquid chromatographic separations followed by spectrophotometric [5–7] or electrochemical detection [3]. In the methods cited above the metabolites were only partly included. Because of the need to monitor all active and major metabolites, respectively, we decided to improve an HPLC-method with UV-detection after solid phase extraction based on already published methods [3,5].

We describe a newly developed solid-phase extraction method for plasma samples combined with RP-HPLC and UV-detection in detail. The method developed for plasma could be applied to urine samples without any change in any parameter.

* Corresponding author. Tel.: +49 551 398582; fax: +49 551 3912767.
E-mail address: imeineke@med.uni-goettingen.de (I. Meineke).

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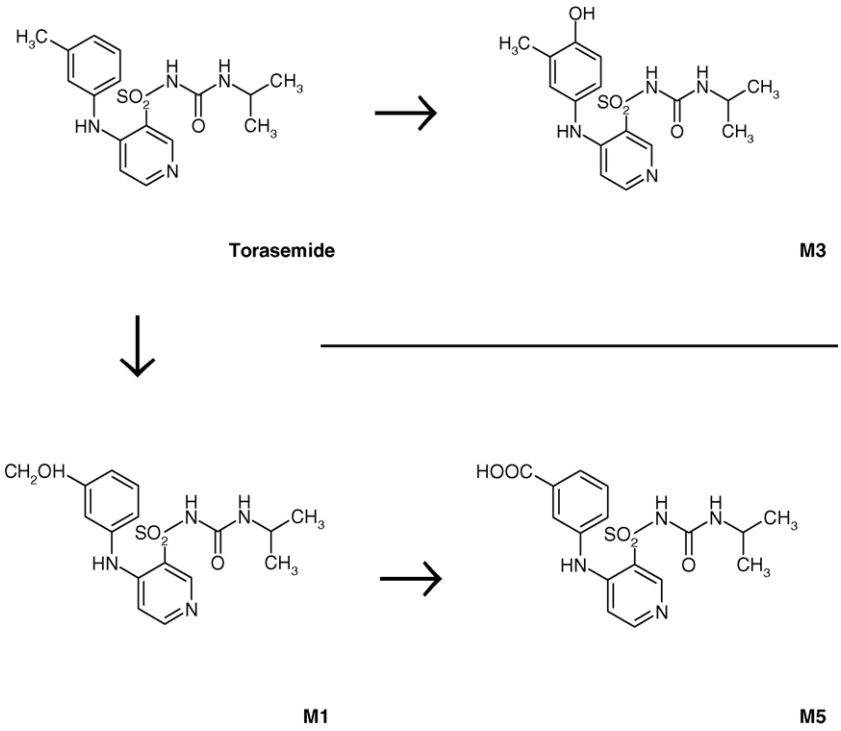

Fig. 1. Biotransformation of torasemide by the metabolic enzyme CYP2C9.

2. Material and methods

2.1. Chemicals and reagents

Torasemide and its metabolites M1 and M3 as free bases together with the metabolite M5 and the internal standard, a trifluoro derivate, as hydrochlorides (Fig. 2) were all kindly supplied by Roche Pharma (Mannheim, Germany). Methanol and acetonitrile were from J.T. Baker, phosphoric acid (85%) and perchloric acid (70%) were from Merck. The chemicals were used without further purification. In all experiments doubly distilled water was used.

For the sample preparation Bond Elut-C_2-cartridges (500 mg) from Varian (Harbor City, USA) were employed.

2.2. Apparatus

The chromatographic system consisted of a Merck-Hitachi model L-7100 pump, a Merck-Hitachi model L-7200 auto sampler and a Merck-Hitachi model L-7400 UV detector (all VWR, Darmstadt, Germany) set at 290 nm. The detector signal was processed on a personal computer equipped with a D 700 data station.

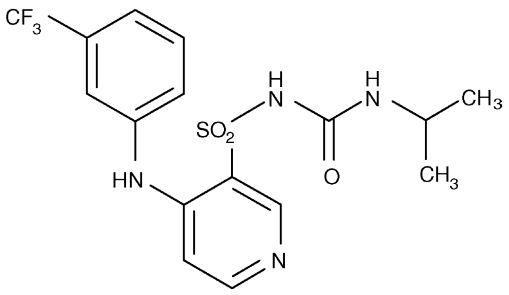

Fig. 2. Internal standard CF_3-torasemide.

For the separation a LiChroCART® CN-cartridge (125 mm × 4 mm, 5 μm particle size, Merck KGaA, Darmstadt, Germany) with a corresponding pre-column (LiChrospher® 100 CN, 4 mm × 4 mm, 5 μm particle size, Merck KGaA, Darmstadt, Germany) was used. The mobile phase was prepared of perchloric acid (0.02 M, pH 2.5, adjusted with sodium hydroxide, 3 M), and acetonitrile (90/10, v/v). The flow rate was kept at 1.5 ml/min at ambient temperature which resulted in a backpressure of typically 90 bar.

2.3. Study design and patient samples

The influence of variations in the genes coding for the metabolic enzyme CYP2C9 on the concentration-time-profiles and the urinary excretion of loop-type diuretics was investigated in a phase IV-study.

The test persons were female or male healthy adults in the age from 19 to 68 years and with a weight between 60 and 80 kg and a body-mass-index between 18 and 25 kg/m^2. Since 2 days before taking the drug, the test persons were subjected to standard low-fat and low-salt diet conditions. Taking any kind of medication was not allowed. Each test person took a single oral dose of 10 mg torasemide of the pharmaceutical product Unat® from Roche.

Samples of blood and urine were taken immediately prior to the dose, and 0.5, 1, 2, 3, 4, 6, 8, 10 and 24 h, respectively, after the dose. The plasma samples were collected in test tubes coated

with ethylene diamine tetraacetic acid as anticoagulant and kept frozen at −20 °C until analysis.

2.4. Sample preparation

After thawing, the samples were briefly treated in an ultrasonic bath. A 1 ml aliquot of each plasma sample was then transferred into a polyethylene centrifuge tube and 50 μl of a stock solution of the internal standard (500 ng) and 0.5 ml of 0.5 M phosphoric acid was added. The sample was consequently applied to a C_2 extraction cartridge which was preconditioned with 2.0 ml methanol and 1.0 ml 0.5 M phosphoric acid. The cartridge was washed in succession with 0.5 ml 0.5 M phosphoric acid, 0.5 ml distilled water and 1.5 ml methanol and finally eluted with 2.0 ml of a mixture of methanol/water (75/25, v/v) into a glass test-tube. The eluate was dried under a gentle stream of nitrogen at a temperature of 40 °C. The residue was reconstituted in 150 μl of a mixture of water/acetonitrile (90/10, v/v). As a rule, 50 μl was chromatographed.

2.5. Calculations and calibration

Stock solutions of torasemide and its metabolites M1, M5 and M3, containing 100, 10 and 1 μg/ml, respectively, were prepared in methanol/water (50/50, v/v). Stock solutions of the internal standard containing concentrations of 1 mg/ml and 10 μg/ml, respectively, were also prepared in methanol/water (50/50, v/v). Calibration samples were made from bovine serum by addition of 500 ng of the internal standard and appropriate aliquots of stock solution to give concentrations at 20, 30, 50, 100, 500 and 1000 ng/ml. A complete set of calibration samples was prepared daily.

The ratio of peak areas (analyte/CF_3-torasemide) were used for the calculation of calibration functions by least-squares linear regression. The inverse squared concentrations were used as weights. Absolute recoveries were calculated by comparing peak areas after injection of known volumes of stock solution with those of calibration samples. Concentrations in ng/ml can be converted into nmol/l by multiplying with a factor 2.874×10^{-3} for torasemide, 2.474×10^{-3} for M1, 2.646×10^{-3} for M5 and 2.857×10^{-3} for M3.

2.6. Quality controls

Two quality control (QC) samples containing 50 and 500 ng/ml, respectively, of each, torasemide, M1, M5 and M3 were included in each series of samples. The control samples were prepared in 20 ml batches, divided in aliquots of 1.2 ml each and kept frozen together with the unknowns until analysis.

3. Results

Under the chromatographic conditions described in the methods section a complete separation of the analytes torasemide, M1, M3, M5 and the internal standard CF_3-torasemide was possible (3B). The retention time for torasemide varied between

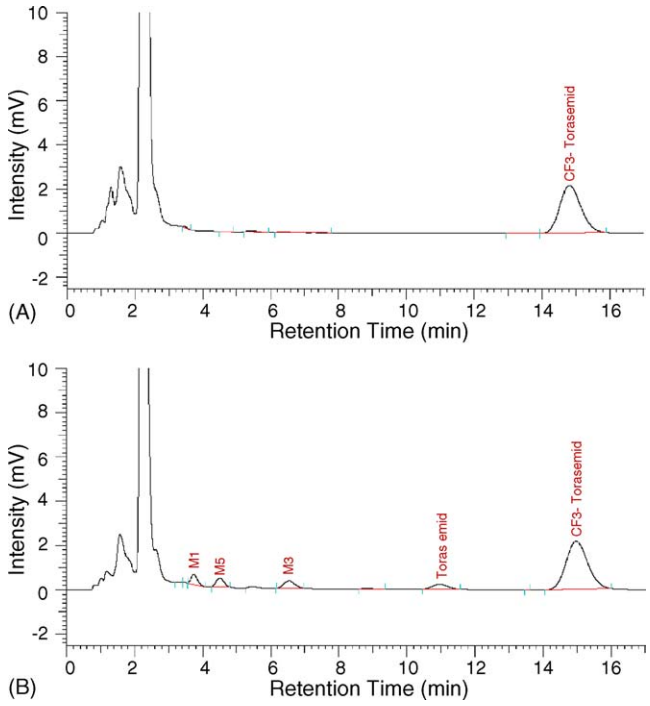

Fig. 3. (A) Chromatogram of blank plasma spiked with 500 ng/ml of internal standard; (B) chromatogram of a calibration sample spiked with 30 ng/ml of M1, M5, M3, torasemide and 500 ng/ml of the internal standard CF_3-torasemide after work-up.

10.30 min and 11.40 min, for M1 between 3.55 min and 3.95 min, for M5 between 4.23 min and 4.73 min, for M3 between 6.21 min and 6.73 min and for the internal standard between 14.10 and 15.35 min.

Using the work-up procedure with solid-phase-extraction the analytes could be isolated from plasma nearly free of endogenous material (Fig. 3A). Only metabolite M3 could interfere with endogenous material but in plasma it normally appears in quantities ≤2% of the parent [3] which is well below the quantification limit (v.i.). Higher concentrations, however, would be easily detectable and quantifiable. In the analyzed patient samples torasemide concentrations between 2226 and <20 ng/ml, M1 concentrations between 159 and <20 ng/ml, and M5 concentration between 420 and <20 ng/ml were found. The metabolite M3 was not detected in any plasma sample. Fig. 4 shows chromatograms of typical patient samples prior to the dose and at 0.5 h after dosage. The peaks correspond to 397.6 ng/ml torasemide, 51.5 ng/ml M1 and 54.8 ng/ml M5 after 0.5 h.

Absolute recoveries of all substances were on average greater than 80%. The calibration functions of all analytes were linear within the concentration range with average slopes of 0.00254 for torasemide (range 0.0021–0.0027), 0.00244 for M1 (range 0.0023–0.0026) and 0.00248 for M5 (range 0.0024–0.0026) and average intercepts of −0.00629 for torasemide (range −0.0209 to 0.0343), −0.00891 for M1 (range −0.0154 to −0.0032) and −0.00465 for M5 (range −0.0119 to 0.0213), respectively. Mean correlation coefficients of 13 calibration curves were calculated as 0.99804 (±0.00312) for torasemide, as 0.99415 (±0.00616) for M1 and 0.99920 (±0.00146) for M5, respectively.

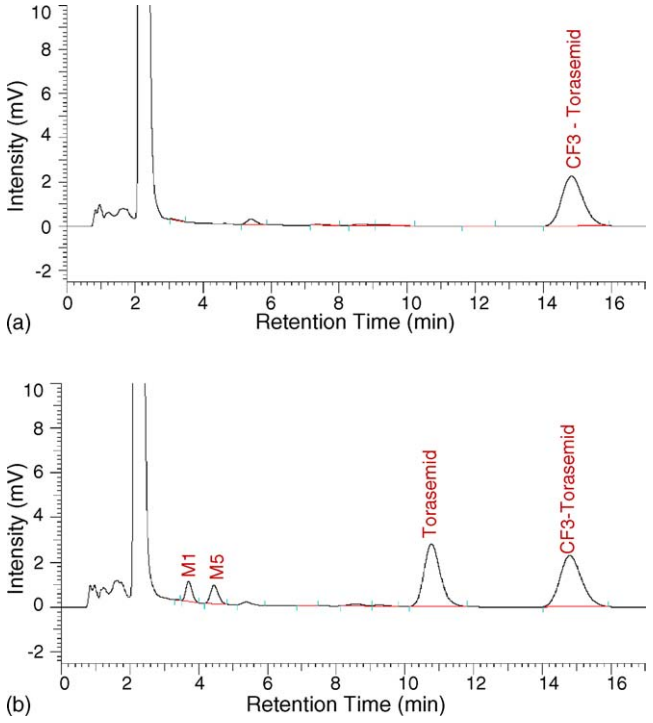

Fig. 4. (A) Chromatogram of a patient sample prior to the dose; (B) chromatogram of a patient sample 0.5 h after a 10 mg dose. The calculated concentrations were 397.6 ng/ml torasemide (peak at 10.79 min), 51.5 ng/ml M1 (peak at 3.71 min) and 54.8 ng/ml M5 (peak at 4.45 min).

Limit of detection and limit of quantification were not formally assessed. Injection of decreasing concentrations (20–1 ng/ml) were performed to collect information with regard to the sensitivity of the system. These experiments revealed that 10 ng/ml could be always distinguished from blank. Therefore, the limit of detection is below 10 ng/ml for all compounds. Eventually, it was decided to establish a calibration range from 20 to 1000 ng/ml. As a consequence results below the lowest calibrator were reported "<20 ng/ml" which was effectively the limit of quantitation. Thus, in all subjects ($n = 36$) concentrations were above LOQ for at least 10 h (torasemide, M5) while for M1 concentration above LOQ were seen for two, occasionally 4 h.

In order to estimate intraday- and interday-reproducibility a series of five repeated measurements at the 50 ng/ml- and the 500 ng/ml level were made. The results are given in Tables 1 and 2.

Table 1
Intraday-reproducibility and accuracy

Analyte	Spiked concentration (ng/ml)	Found concentration mean ($n = 5$) (ng/ml)	Relative standard deviation (%)
Torasemide	500	507.11	9.21
	50	48.88	15.64
M1	500	574.11	5.40
	50	46.98	12.46
M5	500	575.04	5.65
	50	43.69	13.56

Table 2
Interday-reproducibility and accuracy

Analyte	Spiked concentration (ng/ml)	Found concentration mean ($n = 5$) (ng/ml)	Relative standard deviation (%)
Torasemide	500	511.1	5.18
	50	46.8	6.46
M1	500	530.96	6.79
	50	52.31	2.01
M5	500	507.32	6.68
	50	49.74	8.52

Based on the calibration series mentioned above bias and precision of the assay were 4.1 and 8.3%, respectively, for torasemide at the 20 ng/ml level and 4.1 and 10.1% at the 1000 ng/ml level. The M1 metabolite had bias and precision of 1.2 and 4.4%, respectively, at the 20 ng/ml level and 5.0 and 10.6% at the 100 ng/ml level. For the other metabolite, M5, corresponding values were 1.0% bias and 5.3% precision at the 20 ng/ml level with 0.5 and 5.8% at the 1000 ng/ml level, respectively.

After a working-up cycle the solid-phase extraction cartridges were washed with methanol and then used again four times. An increase of interferences was not observed with reused cartridges.

4. Discussion

The developed cleanup procedure and the chromatographic system were applied to approximately one thousand plasma samples obtained from the study which was described above.

The main challenge in the development of an HPLC-method for torasemide and its metabolites consisted in the fact, that the polarities of the metabolites M1 and M5 are very similar but much more polar than the parent compound.

On a C_{18}-column with mobile phases consisting of phosphoric acid and acetonitrile as described previously [5], no eluent composition could be found to separate all compounds in an acceptable time (<20 min). Neither the substitution of the phosphoric acid in the mobile phase with acetic acid nor the change of organic modifier to methanol or addition of triethylamine could improve the separation. The use of a CN-column allowed the complete separation of all substances including the internal standard within 17 min. Various buffers of different concentration levels were tested. The optimal composition of the mobile phase contained 90% of a 0.02 M perchloric acid with a pH of 2.5 and 10% acetonitrile.

The quality of separation of the metabolites degraded slightly by increasing the pH-value only to 3.0 or by increasing the organic part of the mobile phase. By decreasing the pH-value or increasing the buffer component of the mobile phase the retention time of torasemide increased dramatically. By using phosphoric acid in the same concentration instead of perchloric acid, retention times decreased while the quality of separation was not affected. However, the peaks of the metabolites M1 and M5 coeluted with the front peak.

Solid-phase extraction methods for torasemide and its metabolites have been previously described in the literature. The analytes are not retained by CN-, silica- or NH-sorbents while samples are not sufficiently cleaned up by cyclohexyl-, C_{18}- or C_8-sorbents [5]. Liquid–liquid extraction was not attempted because of the very different chemical characteristics of the substances [3]. Considering the polarities of torasemide and its metabolites a high extraction yield of all substances with a simple extraction procedure could not be expected [3].

In contrast using C_2 cartridges with 500 mg sorbent made it possible to process the samples in three steps. With 0.5 ml of 0.5 M phosphoric acid an acidic environment is created to retain the analytes on the cartridge. Afterwards water soluble sample components were removed with 0.5 ml doubly distilled water. In order to obtain samples with a minimum of endogenous material a third wash step with an organic component was required which resulted in nearly interference-free chromatograms. Different volumes of trichloromethane, dichloromethane, methanol and mixtures of methanol and water were investigated for this step. With trichloromethane and dichloromethane endogenous material was not removed completely, so interferences with compounds of interest were still present. On the other hand with mixtures of methanol and water tested at compositions at (50/50, v/v), (60/40, v/v), (70/30, v/v), (80/20, v/v) and (90/10 v/v) the samples could cleaned up satisfactorily but the volumes to elute the substances became unacceptably high. With a volume of 1.5 ml methanol the bulk of endogenous material could be removed and the analytes could be concentrated at the bottom of the extraction cartridge without pre-eluting any analytes. As elution solvent the same mixtures of methanol and water as aforementioned were tested. It turned out that a volume of 2 ml of a mixture of methanol/water (75/25, v/v) represented the optimal composition to elute all substances with recoveries above 80% on average in mostly interference-free chromatograms. Only metabolite M3 could interfere with endogenous material but in plasma it normally appears in quantities $\leq$2% of the parent [3] which is located under the quantification limit. But if there were significant differences M3 would had been both, detectable and quantifiable.

Whether the cartridges employed in the liquid–solid sample preparation step can be used for more than five times has not been investigated. However, there are no reasons why the cartridges should not be used more often in order to reduce costs.

The method developed and validated for the analysis of plasma samples was also applied to a number of urine samples from the same study. Preliminary results indicate that the method can also be used for this matrix without any changes. Analytes were recovered in high yield and the interference-free chromatograms could easily be quantified.

A full report of the results from the clinical study has been published elsewhere [8].

5. Conclusion

In conclusion, we have reported a robust, fast and reproducible method for the determination of torasemide and its metabolites in plasma after the administration of therapeutic doses. Because of the re-use of the solid-phase extraction cartridges this method helps in saving costs and reduces waste.

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