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The determination of torasemide and metabolites in plasma by high-performance liquid chromatography

E. BESENFELDER

Boehringer Mannheim GmbH, Chemical Research Division, Bioanalytical Department, D-6800 Mannheim 31, Sandhofer Str. 116, West Germany

Abstract: A high-performance liquid chromatographic method for the determination of torasemide and two active metabolites is described. The assay uses a reversed-phase gradient system and UV-detection. Sample preparation includes deproteinisation and liquid–solid extraction incorporating an internal standard. Data on recovery, detection limits, precision and accuracy are presented. The presence of an unidentified metabolite also is reported.

Keywords: *Reversed-phase chromatography; liquid–solid extraction; torasemide; active metabolites; plasma drug levels.*

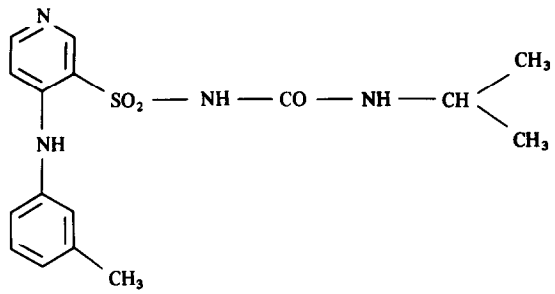
Introduction

Torasemide (I) or 1-Isopropyl-3-(4-m-toluidino-3-pyridyl)sulfonylurea, is a new potent diuretic agent [1]. In dog and rat it is 5–10 times more potent than furosemide on a weight basis. In human volunteers it has a rapid onset of action with a peak effect lasting 2–3 h. Torasemide increases sodium and chloride ion excretion and water clearance, acting essentially on the ascending limb of the loop of Henle. The kaliuretic and phosphaturetic effects are much less pronounced. In order to investigate the pharmacokinetic parameters a method based on high-performance liquid chromatography (HPLC) for the determination of torasemide has been developed [2]. This method utilizes liquid–liquid extraction and determines the unchanged drug in serum. However, in man torasemide undergoes metabolism essentially via the hydroxylation pathway. Two metabolites, M 1 (II) and M 3 (III), are known, each of which also exhibits diuretic potency. This observation led to the development of a HPLC assay for the simultaneous determination of torasemide and its principal metabolites. For the determination of plasma levels the method employs an internal standard (IV),liquid–solid extraction after protein precipitation and reversed–phase liquid chromatography with UV-detection.

Experimental

Reagents

Analytical grade reagents were used as supplied by the manufacturers. Torasemide, M 1, M 3 and the internal standard were synthesized in the authors' laboratories.



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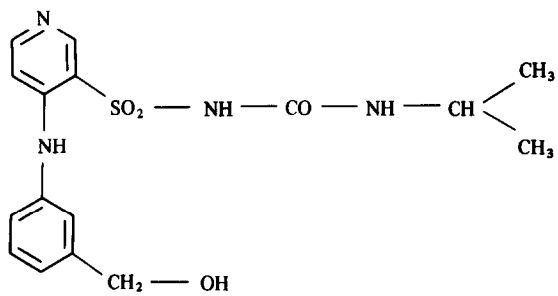

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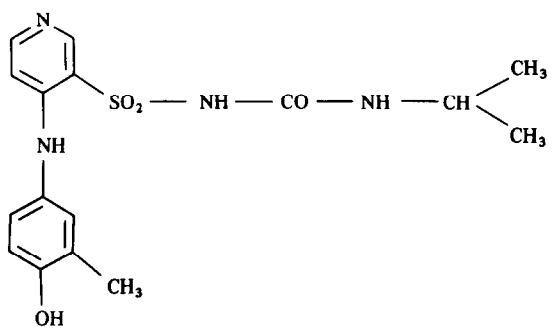

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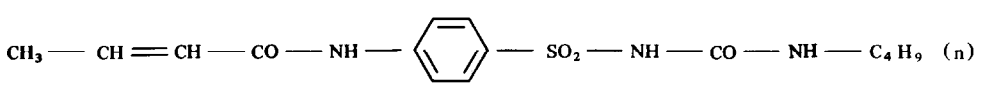

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Chromatography

A Hewlett–Packard HP 1084 B chromatograph was used; the column, 250 mm × 4.6 mm i.d., (Knauer) was slurry-packed with Nucleosil 10 C-18 (Machery & Nagel). The variable wavelength detector was operated at 290 nm; the flow rate was 2 ml min^{-1} under gradient elution conditions. The solvent temperature was 35°C, the oven temperature 45°C, the injection volume 60 µl. The eluents were: solvent A, acetonitrile–0.01 M sodium dihydrogen phosphate (10:90, v/v) at pH 3.0; solvent B, acetonitrile–0.01 M Na H_2PO_4 (50:50, v/v) at pH 3.0. The conditions were: initially, 22% B; t = 5 min, 40% B; t = 10 min, 60% B; t = 15 min, 75% B; t = 20 min, 22% B. Total analysis time was 25 min.

Procedure

1 ml of plasma was mixed with 0.1 ml of internal standard (IV) (5 mg l^{-1} in water) and 0.1 ml of $HClO_4$ (50:50, v/v). The mixture was stirred vigorously on a vibromixer for 30 s, then centrifuged at 2000 **g** for 5 min. The precipitate was extracted twice with 0.5 ml of 0.2 M KH_2PO_4 buffer (pH 3.0) and the combined three supernatants adjusted to pH 3.0 by addition of *ca* 170 μl of 2 M KOH and 1 ml of buffer solution. The solution was transferred to a Bond-Elut Si disposable solid phase column with the vacuum switched off. The sample was then aspirated until dry and rinsed with 0.5 ml of deionized water. Elution of the isolate was performed with 2 × 2 ml of methanol–methylene chloride (50:50, v/v). The combined eluates were evaporated under a stream of nitrogen at 40°C. The dry residue was resuspended in 100 μl of solvent A and centrifuged for 2 min at 3000 **g**. A 60 μl volume of the supernatant was injected onto the column.

Calibration graphs were prepared by chromatographing plasma samples to which known amounts of torasemide and metabolites had been added. Peak height ratios of torasemide or metabolite relative to internal standard were plotted against the known concentrations.

Figure 1
Chromatograms of a blank serum obtained using Nucleosil 10 C-18 (250 mm × 4.6 mm i.d.) column with acetonitrile–0.01 M NaH_2PO_4 gradient elution and UV detection at 290 nm.

Results

Specificity

A typical oral dose of torasemide is 40 mg. For such a dose the plasma concentrations are expected to be in the range 2000–3000 ng ml^{-1} for torasemide, 100–150 ng ml^{-1} for metabolite M 1 and 70–120 ng ml^{-1} for M 3. Figure 1 shows a typical chromatogram of a blank plasma sample. As can be seen it is free from interfering substances, at the elution times for the drug and its metabolites, as indicated in Fig. 2. Figure 3 shows the chromatogram of a volunteer's sample 2 h after a single oral dose of 40 mg torasemide. The signal at run time 4.13 min (x in Fig. 3) seems to be a metabolite peak, because shortly after application it appears with first increasing, then decreasing peak height. Furthermore it seems to be excreted into urine. The chemical structure has yet to be identified.

Evaluation

By measuring six samples of a given concentration the value of the mean and standard deviation for one particular batch were calculated as a measure for "within-run" accuracy and precision.

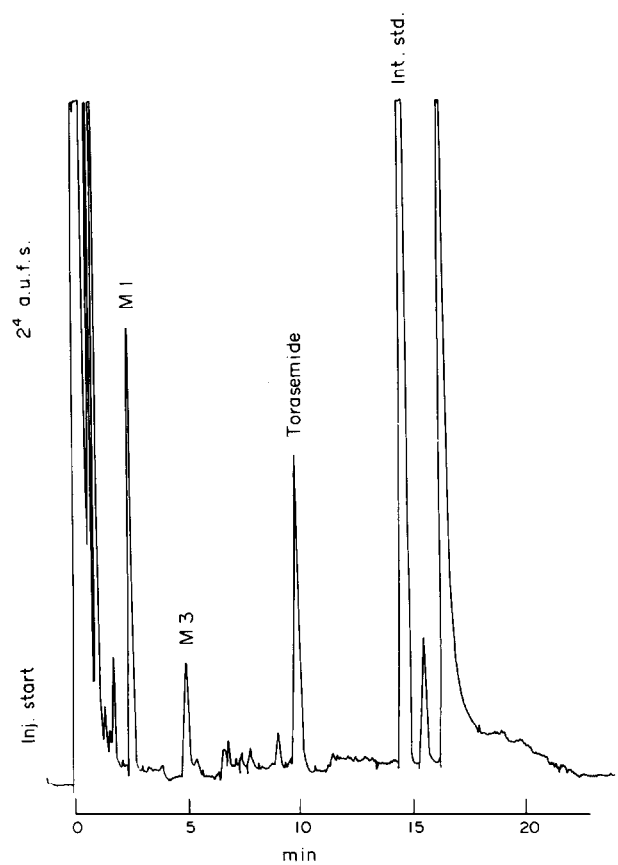

Figure 2
Chromatograms of serum containing torasemide (500 ng ml^{-1}), M 1 (500 ng ml^{-1}), M 3 (1000 ng ml^{-1}) and internal standard (500 ng ml^{-1}), conditions as in Fig. 1.

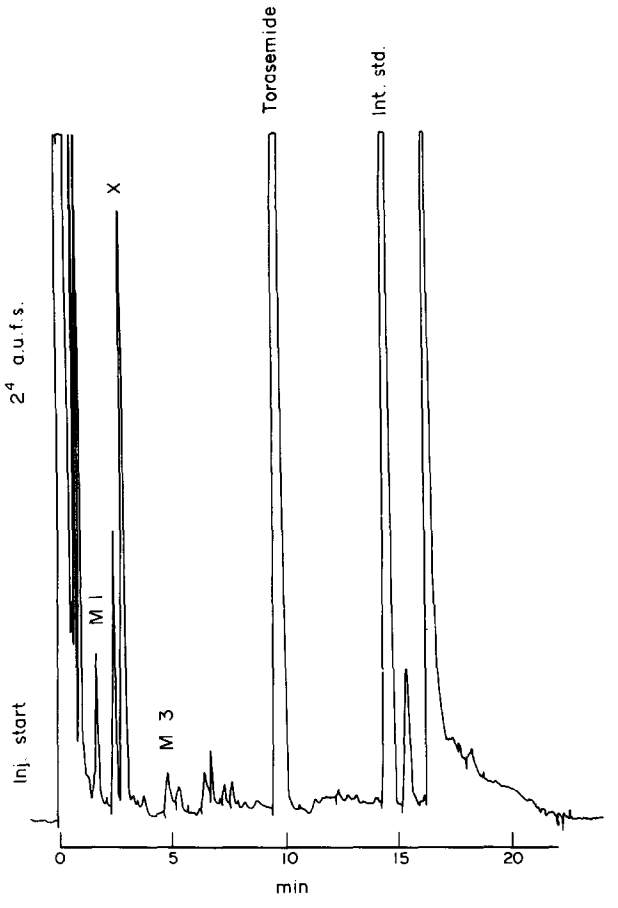

Figure 3
Chromatogram of serum of a volunteer, 2 h after oral ingestion of 40 mg torasemide. X represents a potential metabolite, conditions as in Fig. 1.

The mean value of five successive batches — corresponding to a total number of 30 samples — and the corresponding standard deviation were considered as a measure for "between-run" precision and accuracy.

Table 1 shows the results of 5 batches with plasma samples spiked with either 500 or 1000 ng ml^{-1} of torasemide or metabolites M 1 and M 3.

Recovery

The amounts of torasemide M 1 and M 3 recovered after the extraction of a plasma sample were calculated by comparing the peak areas of a standard solution (N = 5; torasemide, M 1 and internal standard: 5000 ng ml^{-1}; M 3: 10000 ng ml^{-1}) with the peak areas obtained by analysis of a plasma sample with known torasemide M 1 and M 3 concentrations (N = 10;torasemide, M 1 and internal standard: 500 ng ml^{-1}; M 3: 1000 ng ml^{-1}).

Table 1
Determination of torasemide and metabolites. Data illustrating the precision and accuracy of the LC method

Substance	Batch No. 1	2	3	4	5	Mean	SD*	RSD† (%)
M 1	444	540	535	429	490			
(500 ng ml^{-1})	444	533	510	434	492			
	483	493	526	461	470	482	39	8
	403	479	547	440	490			
	407	502	(628)	464	501			
	480	485	541	418	475			
mean	444	505	532	441	486			
SD	34	25	14	18	12			
RSD (%)	8	5	3	4	2			
	1007	1008	1434	893	965			
	1054	1057	1248	893	1069			
M 3	1385	1051	1434	967	902	1096	199	18
(1000 ng ml^{-1})	919	1095	1360	967	902			
	1116	1109	(1601)	998	986			
	971	1058	1546	935	965			
mean	1075	1063	1437	942	965			
SD	166	36	127	43	62			
RSD (%)	15	3	9	5	6			
	477	543	517	482	505			
	472	577	496	438	516			
	520	535	526	447	505	502	37	7
Torasemide	494	525	543	434	502			
(500 ng ml^{-1})	628	532	534	445	521			
	596	514	534	415	526			
mean	491	538	525	444	513			
SD	22	22	17	22	10			
RSD (%)	4	4	3	5	2			

*SD = standard deviation.
†RSD = relative standard deviation (%).

The recovery of the internal standard had been determined previously in an identical fashion. The recoveries found for torasemide, M 1 and M 3 were 77 ± 8, 100 ± 11 and 58 ± 7%; for the internal standard 44 ± 4%.

Linearity

To check the linearity of the method, spiked normal human serum was assayed in the range between 0 and 5000 ng ml^{-1} at 7 concentrations. The resulting correlation was linear for each substance.

The regression data ($y = bx + a$) were:

$b = 1624$, $a = -12$ and $r^2 = 0.9973$ for torasemide,
$b = 2418$, $a = -24$ and $r^2 = 0.9972$ for M 1 and
$b = 9364$, $a = -95$ and $r^2 = 0.9969$ for M 3.

The detector sensitivity was 2^7 a.u.f.s.

Detection limit

Six individual standard solutions of each substance of two relatively low concentrations were assayed (torasemide 40 and 60 ng ml^{-1}, M 1 and M 3 60 and 100 ng ml^{-1}) and the value of the mean and the standard deviation were calculated. The mean limit of detection (DL) was obtained according to the following equation:

$$DL = X_B + \frac{3sn + (3sn - 1) +,\ldots, + 3s}{n},$$

where X_B is the mean value of the blank signal (if any), sn is the standard deviation of an individual concentration range assayed and n is the number of concentration ranges assayed.

Based on reference calibration curves from 0–200 ng ml^{-1}, the limit of detection was found to be 40 ng ml^{-1} for torasemide and 15 ng ml^{-1} for M 1 and M 3, respectively. The detector sensitivity here was 2^3 a.u.f.s.

Discussion

With the described method several hundreds of plasma samples have been analysed. It has proved to be reliable and precise and well suited to pharmacokinetic and bioavailability studies.

Figure 4 shows the plasma concentration profiles of a volunteer after a single oral dose of 40 mg torasemide. Considering the different concentration scales for torasemide on one hand and metabolites M 1 and M 3 on the other, both metabolites show a very low concentration/time profile compared to the parent drug. Much higher concentrations are to be expected for the hitherto unidentified metabolite, which seems to appear in considerable amounts.

Work is in progress to isolate, identify and quantitate this new metabolite; information which will be presented at a later date.

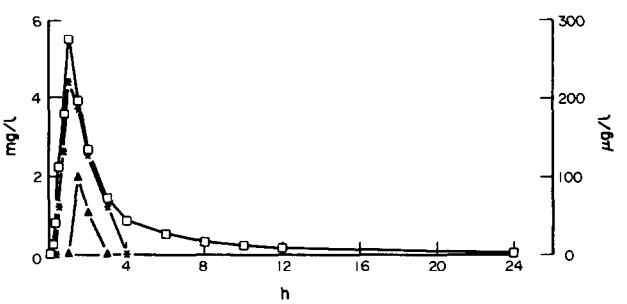

Figure 4
Concentration profile of torasemide, M 1 and M 3 in plasma after a single 40 mg oral dose of torasemide. □, Torasemide (mg l^{-1}); *, metabolite M 1 (µg l^{-1}); ▲, metabolite M 3 (µg l^{-1}).

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References

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