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Implications of Intraindividual Variability in Bioavailability Studies of Furosemide

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Summary. Intrasubject variation in bioavailability (rate and extent) and disposition of furosemide 40 mg was investigated using a repeated, randomized, double-blind cross-over study in 8 healthy subjects. Two generic tablet formulations (Lasix and Furix) and intravenous furosemide were compared on 6 separate days. Extensive intrasubject variability after oral administration was observed in AUC, mean absorption time (MAT) and urinary excretion. The variability (error variance) within the dosage forms was as large as that between the two generics. These variations most probably depended on the absorption process, since the repeated i.v. doses showed only marginal intrasubject variability. Absolute bioavailability was 56% for Lasix and 55% for Furix (AUC). The range was 20 to 84% between individuals and the maximal range within one individual was 20 to 61%. Confidence interval and Bayesian analysis showed a high probability of non-equivalence not only between but also within the generics when the separate cross-over experiments were analyzed (8 observations). When extending the analysis to 16 observations, bioequivalence was demonstrated for the two generic tablets. Rate of absorption, quantified as MAT, was 128 min for Lasix and 98 min for Furix (16 observations). Since MAT was significantly longer ($p < 0.001$) than the mean residence time after the i.v. dose (57 min), absorption was evidently the rate-limiting step in the overall kinetics of oral furosemide. Intraindividual variation in absorption is a confounding factor in bioavailability studies of furosemide using limited numbers of subjects. This is important to consider when designing and evaluating bioavailability studies for drugs showing these variations.

Key words: furosemide; bioavailability, generic tablet formulations, intrasubject variability

The recognition of drug bioinequivalence in the early 70's for generic drugs identified drug bioavailability as a practical problem on which success or failure of drug therapy frequently depends [1, 2]. The problem was also recognized by drug regulatory authorities as generic products represent an important part of the drug market. Regulatory bodies have therefore issued regulations to ensure bioequivalence of generic drugs [3, 4].

Methods for comparative bioavailability studies in man comprise cross-over designs in randomized complete or incomplete blocks. These designs aim to optimise the analysis of the results, including adequate statistics [5]. One of the basic assumptions in cross-over trials is that the individual is constant in his/her clearance of the drug from day to day. When comparing different dosage forms it is also assumed that the absorption processes are constant within the individual. Thus, any differences observed should be related to the dosage forms. However, in the vast majority of published (and unpublished) bioavailability studies these assumptions are rarely challenged.

It has been reported that furosemide may exhibit large intrasubject variation in bioavailability [6]. Therefore, a study has been undertaken to identify the presence and nature of intrasubject variability in the bioavailability of furosemide and its implications when comparing two generic dosage forms, using a repeated complete block cross-over design.

Materials and Methods

Subjects

Eight healthy volunteers, 4 men and 4 women, aged 25 to 27 years, and weighing 53 to 82 kg, gave their informed consent to participation in the study, which was approved by the Ethics Committee at the University Hospital of Uppsala. All subjects underwent

routine medical examination including blood pressure, medical history, urine analysis and S-creatinine. Two subjects were infrequent tobacco users; they did not smoke during experimental days.

Design

The bioavailability of two generic tablet formulations of furosemide was compared in a randomized, double-blind cross-over study, in a repeated complete block design. Furix 40 mg, batch no. 91926, and Lasix 40 mg, batch no. 167 WO 83, were used. Intravenous furosemide (Impugan 10 mg/ml) was also administered on two different occasions. Each subject participated in six experiments separated by at least one week. The dose on all occasions was 40 mg.

The experiments started at 7.30 a.m. No drugs were allowed one week before and no alcohol for 2 days before the experimental days. The subjects were also instructed to avoid very salt food in the 24 h before the experiment and until the last urine sample was collected. The drugs were given after an overnight fast. No food was allowed until 4 h after drug administration, when a standardised lunch was served, corresponding to a total Na^+ and K^+ intake of 48 and 30 mmol, respectively. 200 ml tap water was given every hour for 7 h and 100 ml was given with the dose. Seven hours after the dose, the subjects were allowed food and liquid (not alcohol) ad libitum.

Blood samples were taken at regular intervals for 7 h through an indwelling forearm cannula (Venflon) into heparinized Venoject tubes. Intravenous furosemide was administered in the contralateral arm. The blood samples were left in room temperature for 20 min before plasma was harvested. The plasma was frozen and stored at −20 °C until analysed. Urine was collected as voided for 24 h. The urine from 7 to 24 h after the dose was pooled.

Furosemide Analysis

The concentrations of furosemide in plasma and urine were measured by HPLC with fluorimetric detection [7]. 0,25–0,5 ml plasma and 0,1 ml urine were used in the assay. The determination limit of the assay was 20 ng/ml. In plasma, the reproducibility of the assay was ±5,5% (50 ng/ml) and 4,3% (200 ng/ml); in urine it was 5,9% (2 µg/ml). The assay procedure was performed in such a way as to minimize photochemical and acid degradation of furosemide.

Pharmacokinetic Analysis

The following calculations were made:

$$\text{Clearance (CL)} = \frac{\text{Dose}_{\text{i.v.}}}{\text{AUC}_{\text{i.v.}}}$$

Volume of distribution at steady state

$$(\text{Vd}_{\text{ss}}) = \frac{\text{Dose}_{\text{i.v.}} \times \text{AUMC}}{\text{AUC}^2}$$

Absolute bioavailability

$$(\text{F}) = \frac{\text{AUC}_{\text{oral}} \times \text{Dose}_{\text{i.v.}}}{\text{AUC}_{\text{i.v.}} \times \text{Dose}_{\text{oral}}}$$

AUC and AUMC for the individual curves were calculated by the trapezoidal rule up to the last data point. The extrapolated area to infinite time for AUC and AUMC beyond the last data point was estimated by integration.

Extent of Absorption

Comparison of the extent of absorption of the different dosage forms after oral administration was based on AUC and urinary excretion measurements.

Rate of Absorption

Statistical moments [8, 9, 10] were used as a model-independent way of estimating the time course of absorption and disposition of furosemide.

$$\text{Mean Residence Time (MRT)} = \frac{\int_0^\infty \text{tCdt}}{\int_0^\infty \text{Cdt}} = \frac{\text{AUMC}}{\text{AUC}}$$

$$\text{Mean Absorption Time (MAT)} = \text{MRT}_{\text{oral}} - \text{MRT}_{\text{i.v.}}$$

Statistical Analysis

Two way analysis of variance for balanced data (ANOVA) was used to test for differences between dosage forms. To test for bioequivalence, symmetrical confidence intervals according to Westlake [11, 12], and Bayesian analysis according to Rodda and Davis [13], were used. Lasix was used as the reference drug. All results are given as mean ± SD.

Results

Extent of Absorption – AUC

The total AUCs found after repeated oral and intravenous administration of 40 mg furosemide to the 8 subjects are summarised in Table 1. Large inter- and intra-individual variation were found after oral administration. The absolute bioavailability was 56 ± 18% for Lasix and 55 ± 12% for Furix, respectively. The range was 20 to 84% between individuals, and the maximal range within one individual was 20 to 61%.

Data from one subject given each dosage form in a repeated cross-over design are shown in Fig. 1. The plasma concentration-time profiles were totally dif-

Table 1. Total AUC [μg/ml/min] after oral and intravenous administration of furosemide, 40 mg on two different occasions

Subject	Lasix (1)	Lasix (2)	Furix (1)	Furix (2)	i.v. (1)	i.v. (2)
AH	165	162	137	111	229	231
AL	269	235	167	209	386	363
DL	147	109	184	91	277	243
EA	95	108	108	173	312	302
ES	176	184	174	147	326	270
ME	161	207	195	154	239	254
MS	169	242	151	190	358	326
UB	62	128	190	190	317	307
Mean	155	172	163	158	306	287
±SD	60.9	53.9	29.7	41	54.6	45.2

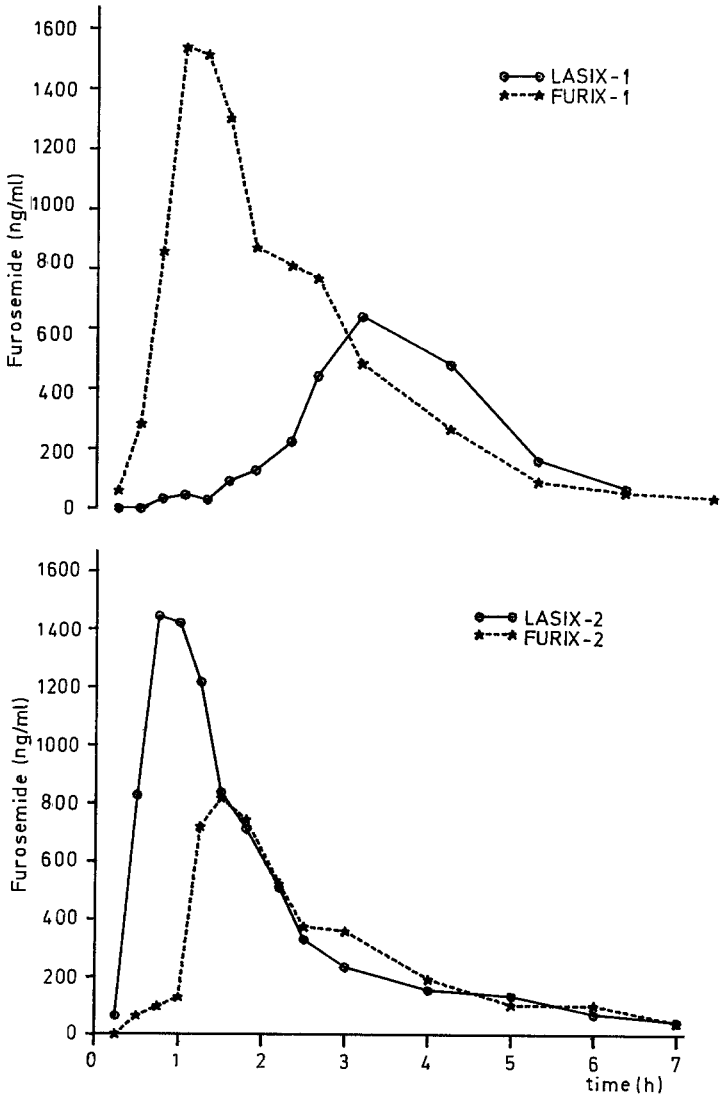

Fig. 1. Plasma concentrations of furosemide in one individual given Lasix and Furix tablets (40 mg) as a single oral doses on two different occasions (repeated cross-over test)

ferent in the two experiments. In the first cross-over experiment (top) Furix showed more complete bioavailability than Lasix, whereas the contrary was found in the second experiment (bottom). Some individuals (two shown in Fig. 2) showed the same absorption from the two dosage forms. An example of extreme intraindividual variation in bioavailability of furosemide from the same dosage form (same batch) is shown in Fig. 3.

Statistical analysis of the AUC data is summarized in Table 2. Due to the large inter- as well as intra-individual variability, no statistically significant difference in AUC could be detected (ANOVA). However, confidence interval and Bayesian analysis

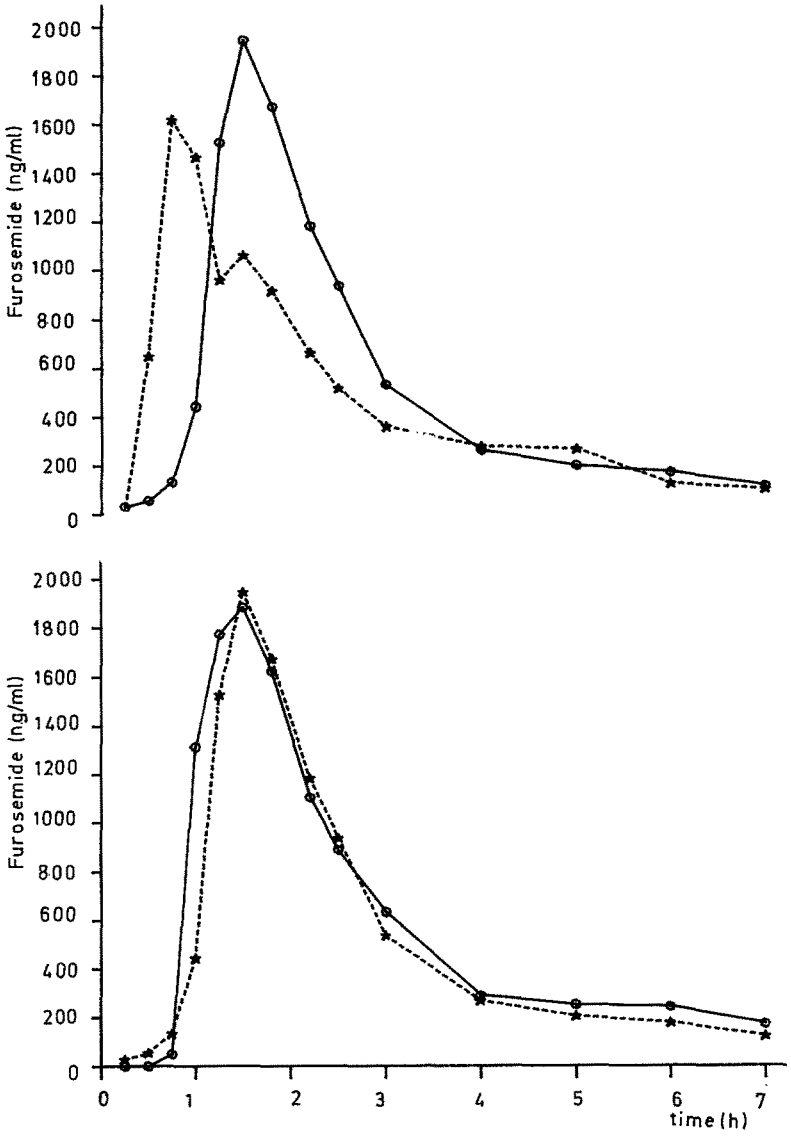

Fig. 2. Plasma concentrations of furosemide in two individuals (top and bottom) given Lasix and Furix tablets as single oral doses on one occasion (cross-over test, second period) O——O Lasix-2; ★——★ Furix-2

indicated a high probability of non-equivalence not only between but also within dosage forms when the separate cross-over experiments were analyzed (8 observations). The relative error variance was of the same magnitude within as well as between dosage forms.

From Table 2 it can be seen that using 16 observations, confidence intervals decreased to 19% and the probability of a true 20% difference between dosage forms was only 6%. Intraindividual variation in AUC after intravenous administration was substantially smaller than after oral administration, the relative error variance being of the order of only 5% of that found after oral dosing.

The maximal deviation of the plasma concentrations of furosemide observed in one individual when 40 mg furosemide was given intravenously at two occasions is illustrated in Fig. 4.

Extent of Absorption – Urinary Excretion

Urinary excretion of unchanged furosemide (24 h) was 28.2 ± 2.2 mg after intravenous administration and 16.2 ± 3.6 and 16.2 ± 3.1 mg after Lasix and Furix, respectively. The absolute bioavailability calculated from urinary excretion data was therefore in good agreement with the bioavailability based on AUC. Urinary excretion (oral) ranged from 7.8 to 23.0 mg between individuals and the maximal range within one individual was 7.8 to 13.0 mg for Lasix and 12.0 to 23.0 mg for Furix, respectively.

Statistical analysis of the urinary excretion results showed the same pattern as the AUC data. Confidence intervals were ± 22% (mean value) between dosage forms and ± 26% (Furix) and ± 23% (Lasix) within dosage forms (8 observations). When extending the analysis to 16 observations the confidence intervals decreased to ± 14% between dosage forms.

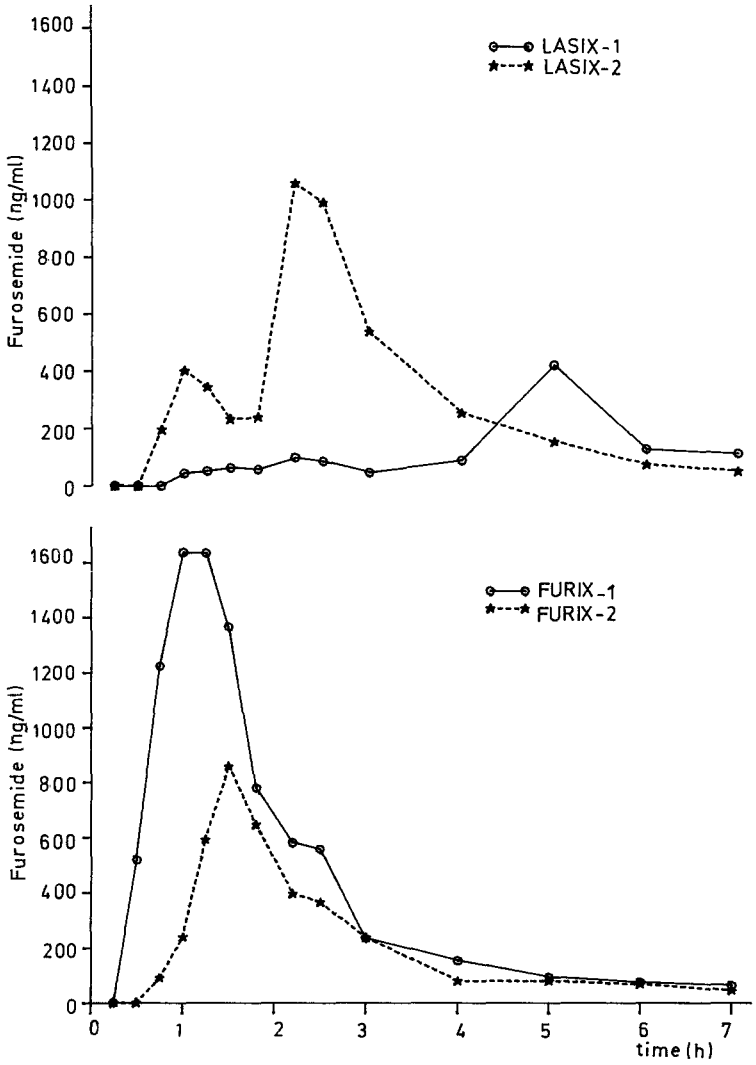

Fig. 3. Examples of intraindividual variation in the plasma concentrations of furosemide in two subjects (top and bottom) given Lasix and Furix tablets, respectively, on two different occasions (repeated cross-over test)

Table 2. Confidence interval and Bayesian analysis of AUC-data following repeated intravenous and oral administration of 40 mg furosemide

Administration	Number of observations	Confidence interval [%]	Probability of a 20% difference [%]
i.v. (1) –i.v. (2)	8	±11	0.1
Lasix (1)–Furix (1)	8	±36	35
Lasix (2)–Furix (2)	8	±28	28
Lasix (1)–Lasix (2)	8	±29	36
Furix (1)–Furix (2)	8	±27	20
Lasix –Furix	16	±19	6

Rate of Absorption

The average mean residence time after i.v. administration of furosemide was 57 ± 11 min (Table 3). The average mean absorption time was 128 ± 55 min for Lasix and 98 ± 29 min for Furix (N.S., 16 observations), respectively.

The areas under the curve for the i.v. dose from the last data point to infinity for total AUC and AUMC were 0.5 to 3.5%, and 3.6 to 21 (mean 11)% respectively. For the oral doses they were 0.8 to 17 (mean 5,8)%, and 3.6 to 48 (mean 15)%, respectively.

In the calculations of the extrapolated AUMC for the i.v. dose, an error was introduced for some individuals when basing the calculations on the final slope (last 3–4 data points). Then, the last 6–7 data points were used in order to stabilize the calculations of total AUMC.

Confidence interval analysis (Table 4) showed a very small confidence interval for the i.v. dose between the 2 treatment days. The MRT for Day 2 was within ±6 min of the value for Day 1. However, when looking at the oral doses, the confidence interval for MAT was much larger (51 to 95 min). The variation for MAT was as large when one dosage form was compared between days (intraindividual variation) as when Lasix and Furix were compared. The large confidence intervals indicated non-equivalence of the rate of absorption of the generic tablets when analyzing the separate experiments. In contrast

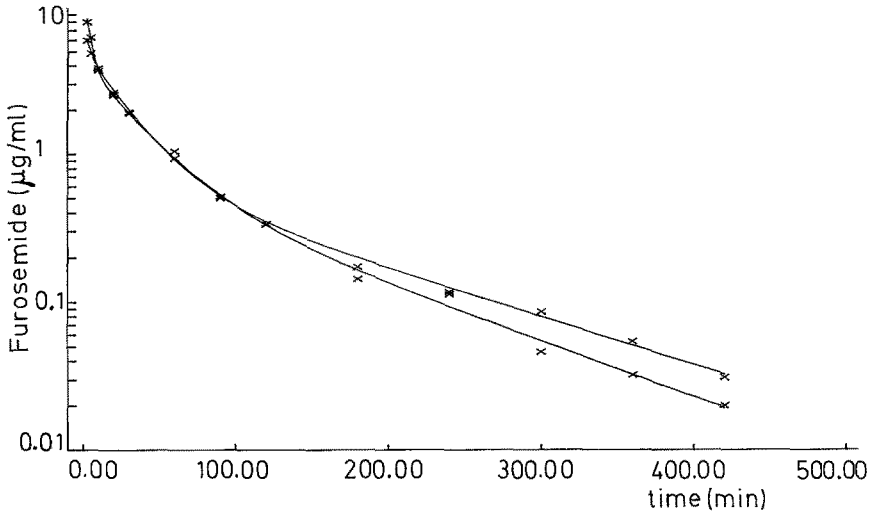

Fig. 4. Example of maximal intraindividual variation in the plasma concentrations of furosemide in one subject given intravenous furosemide (40 mg) on two different occasions

Table 3. Individual mean residence times (i.v.) and mean absorption times of furosemide. In the calculations of MAT, a mean value of $MRT_{i.v.}$ for Days 1 and 2 was used

Subject	Intravenous dose		Lasix		Furix	
	$MRT_{i.v.}$ (1)	$MRT_{i.v.}$ (2)	MAT (1)	MAT (2)	MAT (1)	MAT (2)
AH	70.9	59.2	137	71.9	91.9	117
AL	71.6	73.2	136	118	64.6	93.6
DL	53.1	51.0	185	158	90.9	128
EA	41.3	41.4	173	206	114	86.6
ES	52.7	42.3	69.5	134	98.5	176
ME	49.2	51.5	65.7	72.7	93.7	77.7
MS	60.1	67.0	46.4	117	65.4	77.4
UB	65.5	68.9	242	118	65.8	129
8 Observations						
Mean	58.0	56.8	132	124	85.6	111
±SD	10.8	12.2	67.9	43.8	18.3	33.8
16 Observations						
Mean	57.4		128[a]		98.1[a]	
±SD	11.1		55.3		29.3	

[a] $p < 0.001$ compared to $MRT_{i.v.}$

to extent of absorption, combined analysis ($n = 16$) did not decrease the confidence intervals of the rate of absorption. With the current batches used, Furix seems to be more rapidly absorbed, than Lasix, although conventional ANOVA did not demonstrate significant difference between the 2 tablets.

Urine Volumes

The urinary output (7 h) was 2.21 ± 0.363 l after intravenous administration and 1.90 ± 0.376 l and 2.0 ± 0.488 l after administration of Lasix and Furix, respectively. After oral administration urinary volumes ranged from 1.08 to 2.91 l between individuals, and the maximal range within one individual was 1.56 to 2.41 l (Lasix), and 1.37 to 2.28 l (Furix).

The confidence interval based on eight observations was 15% (mean) between dosage forms and ± 24% (Lasix) and ±21% (Furix) within dosage forms, respectively. When the statistical analysis was extended to 16 observations the mean urinary output after administration of Furix was within ±11% of the mean urinary output after administration of Lasix.

Discussion

The most important finding in the present study is the extensive intraindividual variation both in the rate and extent of absorption of furosemide from two oral dosage forms. Although strictly standardized conditions were employed, and healthy volunteers and the same batches of the different dosage forms were used, the magnitude of the intraindividual variation was in the same range as the interindividual variation. The two dosage forms showed approximately equal within-subject variation, indicating that the dosage forms were "equivalent" in this respect.

The disposition kinetics of furosemide following intravenous administration, eg. volume of distribution at steady-state (7.9 l), plasma clearance (139 ml/min), mean residence time (57 min) and urinary excretion of unchanged drug (70% of dose), was in good agreement with previous reports [14–17]. Thus, the findings are not likely to be due to methodological pitfalls but rather they reflect real variation.

In most individuals, a triexponential decline in the plasma concentration was evident after the i.v. doses. An error can be introduced in calculation of AUMC when there is pronounced multicompart-

Table 4. Confidence interval and Bayesian analysis of mean residence (MRT) and mean absorption times (MAT) following repeated intravenous and oral administration of 40 mg furosemide

Administration	Number of observations	Confidence interval		Probability of a 20% difference [%]
		±[%]	±[min]	
$MRT_{i.v.}$ (1) $-_{i.v.}$ (2)	8	10	6	0.3
MAT Lasix (1)–Furix (1)	8	72	95	[a]
MAT Lasix (2)–Furix (2)	8	41	51	57
MAT Lasix (1)–Lasix (2)	8	43	57	44
MAT Furix (1)–Furix (2)	8	58	50	[a]
MAT Lasix –Furix	16	45	58	[a]

[a] Actual difference exceeded 20%

mental behaviour of the drug [18], depending on difficulties in calculating the extrapolated area. This was also found in the present study. The t × C values may even rise when the final slope declines slowly, thus producing a large error in the AUMC calculations. The AUC for the final slope in the plasma concentration curve for furosemide has been shown to represent only about 10% of the total AUC [7]. Accordingly it was considered justified to use plasma concentration values from the last two phases in calculating the extrapolated area to diminish the potential error in calculation of the AUMC.

The preliminary finding of Dagrosa et al. [6], of extensive intrasubject variation in absorption and/or disposition of oral furosemide in two individuals has been confirmed in the present study. The results of the repeated intravenous administrations showed substantially smaller intraindividual variations than the oral administrations. Thus, the major source of variation is most probably attributable to variation in the rate and extent of absorption.

The mechanisms behind the extensive intrasubject variation in absorption cannot be fully elucidated, since an oral solution was not employed in this study. A contribution of day to day variation in vivo dissolution cannot be ruled out. The FDA has shown that the extent of absorption of furosemide generics is influenced by the in vitro dissolution properties [19]. However, considering the results of Hammarlund et al. [7], of an erratic and variable absorption profile of furosemide, a more likely explanation of our finding is day to day variation in physiological factors affecting gastric emptying and gastric pH. These factors by themselves might influence both the rate and extent of absorption of drugs, as well as the in vivo dissolution profile of a specific dosage form [20].

The erratic and discontinuous plasma concentration-time profiles after oral administration prompted us to use moment analysis to evaluate the rate of absorption. In fact, Hammarlund found it impossible to obtain a meaningful estimate of the rate of absorption using a deconvolution technique (unpublished data). It was possible to confirm the results of Hammarlund et al. [7], that the MRT after intravenous administration was significantly shorter than the MAT after oral administration of Lasix and Furix. This strongly indicates that furosemide kinetics is rate-limited by absorption.

The implications of intraindividual variations in both the rate and extent of absorption for the evaluation of bioavailability studies of furosemide using classical cross-over designs are evident. As seen from Tables 2 and 4, the statistical analysis indicated a large probability of non-equivalence not only between dosage forms but also within the same dosage form! Thus, intrasubject variability contributed to a large extent to the total variability, thereby indicating that the true difference between dosage forms was smaller. It was evident that the two dosage forms could be considered bioequivalent with regard to the extent of absorption, accepting a ±20% difference (16 observations; Table 2). Comparing the different parameters, urine volume showed the smallest confidence intervals. In healthy volunteers, the diuretic effect of furosemide was highly dependent on the water load given to the subjects (unpublished observations). This makes diuresis a less sensitive bioequivalence parameter for detecting variations in the extent and rate of absorption of furosemide.

It could be argued that if a substantially larger number of subjects had been included, the influence of intrasubject variability would have been diminished. This would be true if it be assumed that the actual intrasubject variability had a normal distribution. Using the approach of Rodda and Davis [13], it could be calculated that between 20 to 37 subjects would have been required in a non-repeated design to detect a true 20% difference between the dosage forms with a power of 80% ($\alpha = 0.05$). Thus, to overcome the contribution of intrasubject variability as a confounding factor in the analysis of bioequivalence studies of furosemide, large numbers of subject would have to be included in a classical cross-over design. A repeated design, as used in this study, has the advantage that the magnitude of intrasubject var-

iability can be estimated. As the randomization procedure is identical between the two experiments, 16 observations could be used and were found sufficient to establish bioequivalence. To avoid large subject numbers for practical, economical and ethical reasons, the most appropriate technique for furosemide would be to use a stable isotope-labelled internal standard. This technique totally eliminates intrasubject variability and substantially reduces the number of subjects required [21].

In conclusion, intrasubject variability has been shown to be a confounding factor in bioavailability studies of furosemide based on a limited number of subjects. In general, intrasubject variability can induce different effects in the analysis of bioavailability studies. Actual differences may either be masked or amplified. False differences can also be introduced. This should be kept in mind when designing and evaluating bioavailability studies.

As pointed out by Jack et al. [22], the clinical implications of variability in drug disposition should not be overemphazised. Many drugs show flat dose-(concentration)-response curves and dose titration is a rational approach to limit the effects of interindividual variation. Intraindividual variability, however, can be more difficult to cope with. Such variations have definite clinical implications for drugs with a narrow therapeutic range.

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