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Fully automated gradient elution liquid chromatographic assay of omeprazole and two metabolites*

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Abstract: An automated liquid chromatographic method for the determination of omeprazole and two metabolites in plasma and urine is described. It utilizes the Technicon Fully-Automated-Sample-Treatment-LC system (FAST®-LC). Sample preparation is achieved by air-segmented continuous-flow providing solvent extraction, evaporation to dryness and reconstitution before injection onto a reversed-phase column. The compounds are separated by isocratic or gradient elution with acetonitrile–phosphate buffer mobile phases and quantified by UV-measurements at 302 nm. The limit of determination (relative standard deviation 10–15%) is 50 nmol l^{-1} in plasma (800 μl) and 200 nmol l^{-1} in urine (200 μl). The sample capacity is six or three samples per hour, depending on the elution mode.

Keywords: *Omeprazole; metabolite; reversed-phase chromatography; automation; drug assay.*

Introduction

Omeprazole (5-methoxy-2[[(4-methoxy-3,5-di-methyl-2-pyridinyl)methyl]sulphinyl]-1H-benzimidazole) is a long-acting inhibitor of gastric acid secretion [1]. It appears to act by direct interaction with the proposed proton pump by selectively inhibiting the H^+,K^+-ATPase [2].

Two manual methods for the determination of omeprazole in biological fluids have been published previously [3, 4]. In order to provide the large number of plasma and urine assays required for the documentation of the drug, an automated method is desirable. High sample throughput is achieved in the present study by application of the Technicon Fully-Automated-Sample-Treatment-LC (FAST®-LC) technique to the determination in plasma and urine of omeprazole (**I**) and two metabolites, namely hydroxyomeprazole (**II**) and the sulphone (**III**) (Fig. 1).

Sample preparation is achieved by an air-segmented continuous-flow system providing solvent extraction, evaporation to dryness and reconstitution before injection onto a

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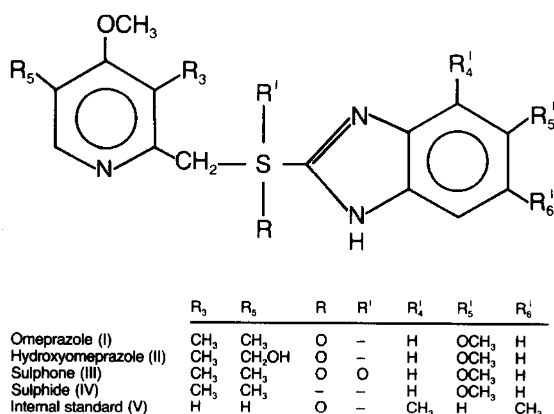

	R_3	R_5	R	R'	R_4'	R_5'	R_6'
Omeprazole (I)	CH_3	CH_3	O	–	H	OCH_3	H
Hydroxyomeprazole (II)	CH_3	CH_2OH	O	–	H	OCH_3	H
Sulphone (III)	CH_3	CH_3	O	O	H	OCH_3	H
Sulphide (IV)	CH_3	CH_3	–	–	H	OCH_3	H
Internal standard (V)	H	H	O	–	CH_3	H	CH_3

Figure 1
Chemical structures of omeprazole, its metabolites and the internal standard.

reversed-phase column. Isocratic elution is used for determination of **I** and **III** and gradient elution when **II** also is of interest.

Experimental

Apparatus

The FAST®-LC system (Technicon Instruments Corp.) used for sample treatment consists of: a sampler, a proportioning pump, a sample processing cartridge, an Evaporation-to-Dryness Module (EDM) and a vacuum pump. The liquid chromatograph used consisted of a FAST®-LC cartridge with a six-port pneumatically activated injection valve provided with a 1.2 ml sample loop, a solvent metering pump (Beckman Model 110 A) and for gradient elution a system controller (Beckman Model 421) working with two solvent metering pumps (Beckman Model 110 A), a variable wavelength UV-detector (LDC SpectroMonitor III) and a computing integrator (Spectra-Physics 4100). The entire system is synchronized by means of a microprocessor. An automated wash of the sample preparation system is activated by another microprocessor, which also shuts off the system after the last assay.

Chromatographic system

The separation column (stainless steel, 150 × 4.5 mm) was packed with Polygosil C18, 5 μm particles (Machery-Nagel & Co.). The analytical column was protected by a guard column (stainless steel, 30 × 4.6 mm) packed with Spheri-5, RP18 (Brownlee Labs Inc.).

The mobile phase was composed of acetonitrile and phosphate buffer, pH 7.7 (I = 0.05). Isocratic elution with 34% acetonitrile was used for the determination of **I** and **III** in plasma and a gradient elution system for all three compounds (**I–III**) in plasma and urine. The gradient profile consisted of two isocratic parts and two linear steps within a period of 20 min. After 3 min with 25% acetonitrile the concentration was linearly increased to 40% during the next 1 min and kept there for 6 min. In the following 5 min the acetonitrile content was decreased to 25%. The system was equilibrated for 5 min before the next injection.

In both systems the compounds were eluted within 10 min using a flow-rate of 1.5 ml

min^{-1}. The eluent was monitored at a wavelength of 302 nm. Quantitative analysis was based on peak height measurements and internal standardization.

Chemicals and reagents

The chloroform, propan-2-ol, methanol and acetonitrile used were of HPLC grade (Rathburn Chemicals, Scotland, UK). Omeprazole (**I**), metabolites (**II–IV**) and the omeprazole analogues were all synthesized by the Department of Organic Chemistry, AB Hässle. The omeprazole analogue H 153/52 (**V**, Fig. 1) was used as internal standard. Omeprazole fulfilled the quality requirements of the Pharmacopoeia Nordica. All other solutions were prepared from analytical grade chemicals.

Extraction solvent. Propan-2-ol–chloroform (25:75, v/v), freshly made every two days and kept in dark bottles.

Solution for pH adjustment. 0.4 M sodium dihydrogen phosphate.

Sample wash solution. Sodium carbonate buffer (pH 9.2, I = 0.1).

Pick-up solution. Acetonitrile–phosphate buffer (pH 7.7, I = 0.05) (10:90, v/v).

EDM wash solutions. Propan-2-ol–chloroform (25:75, v/v) at station 1; ethanol–chloroform (50:50, v/v) at station 2.

Analyte calibration solution. A standard solution containing **I**, **II** and **III** was prepared by dissolving 0.80 mg of each in 20.0 ml of methanol and diluting to 100.0 ml with carbonate buffer (pH 9.2, I = 0.1). The concentration of each compound in the calibration solution was 23 μmol l^{-1}. The solution was kept frozen at −18°C in 2 ml portions in 4 ml sample cups for up to three months.

Plasma standards. An 800 μl sample of human blank plasma was added to each sample cup containing 100 μl of standard solution. This corresponded to a concentration of each compound of 3 μmol l^{-1}. One cup without any standard solution was used as a blank.

Urine standards. A 200 μl sample of human blank urine was added to each sample cup containing 100 μl of standard solution to give a concentration of each compound of 12 μmol l^{-1}. One cup without any standard solution was used as a blank.

Internal standard solution. A 1.0 mg sample of **V** was dissolved in 10.0 ml methanol and diluted to 50.00 ml with carbonate buffer (pH 9.2, I = 0.1). The concentration of the internal standard solution was 64 μmol l^{-1} and was used for plasma samples. For the urine samples a 10.00 ml volume of this solution was diluted to 50.00 ml with distilled water. The internal standard solutions were stored in a refrigerator at +(4–8)°C for up to a week without problems.

Extraction experiments

The distribution ratios (D) were determined for compounds **I**, **II**, **III**, **IV**, and some analogues, between pre-equilibrated solutions of propan-2-ol–chloroform (25:75, v/v)

and phosphate buffer (pH 7.0, I = 0.10). Equal volumes of aqueous and organic phases in centrifuge tubes were mechanically shaken for 30 min at room temperature. The concentration of the compounds in the aqueous phase before and after mixing was determined by liquid chromatography with UV-detection. The concentration in the organic phase was obtained as the difference between the initial and the final concentration in the aqueous phase.

Analytical procedure

Plasma method. The frozen plasma sample was thawed at room temperature and then mixed and centrifuged. A portion of 800 μl was transferred to a 4 ml sample cup containing 100 μl of the internal standard solution (64 μmol l^{-1}). After mixing for 5 s on a Whirlmixer (Fisons), the cups are placed on the sample tray. The first cup and every tenth cup contained a plasma standard.

The following operations were automatically performed under the direction of a microprocessor. The procedure is schematically described in the flow diagram shown in Fig. 2. The sampler was initiated to aspirate the sample for 2 min. The plasma sample was segmented with air and after pH adjustment extracted with the organic solvent in the extraction coil for 4–5 min. An aliquot of the organic phase (50–60%) was separated from the aqueous phase and the air bubbles in a Y-shaped phase separator.

The organic phase was air-segmented and applied to the teflon wire inside the EDM module. Complete evaporation of the extract was achieved by an airstream at 70°C, final traces being removed by vacuum. The residue was reconstituted at the pick-up station, which consists of a 4.5-cm glass tube through which the pick-up solution is pumped countercurrently. As the pick-up solution with the dissolved sample is pumped out of the station with a higher flow-rate, it becomes air-segmented. The air is removed in a debubbler placed in front of the injection valve.

The sample solution is automatically injected onto the chromatographic column. The time for the first injection depends on the lag time of the system, which is usually about 17 min but can vary a little from day to day. The lag time is visually estimated by

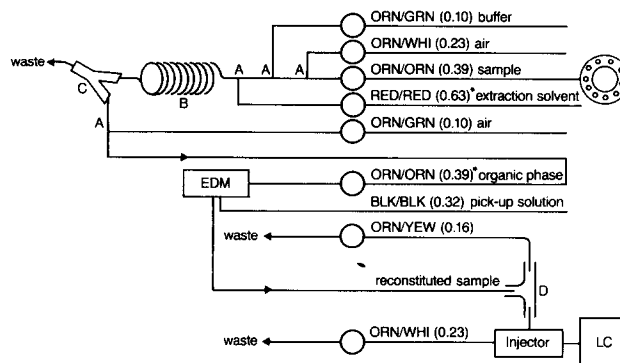

Figure 2
Flow diagram of the sample preparation procedure. Acidflex pump tubes are asterisked. The others are tygon tubes. Flow-rates in ml min^{-1} are given in parentheses. Sample tubing is polyethylene (i.d. 0.87 mm). Transport tubing for organic phase is polypropylene (i.d. 0.87 mm) and between pick-up station and injection valve Kel-F (i.d. 1.28 mm). (A) 1-mm i.d. glass T; (B) extraction coil made from a polypropylene tube 4.5 m × 1.28 mm i.d.; (C) Y-shaped polypropylene phase separator (i.d. 2.0 mm); (D) debubbler made of glass. The hydraulic connections to the EDM wash stations are not shown.

extraction of a picrate solution at acidic pH. The time between samples entering the system must at least exceed the chromatographic analysis time. The isocratic elution mode allows a sample to be aspirated every 10 min compared with every 20 min if the gradient elution system is used.

Urine method. The urine samples were assayed according to the same procedure as the plasma samples, with the following modifications. Only 200 μl urine was used for each assay and a larger volume (700 μl) of the more diluted internal standard solution was used.

Results and Discussion

Extraction

Various aspects must be considered in the choice of an organic solvent for sample treatment in a continuous system like the one used in the present study. Besides a high extraction efficiency, the organic phase has to give a good segmentation in the extraction coil, in order to ensure a reliable phase separation. Other properties, such as volatility, density and surface tension, have to be considered for the evaporation step in the EDM module. Methylene chloride and dichloroethane, pure or in mixtures with propan-2-ol or butan-2-ol, were found to be inappropriate since they gave neither good phase segmentation owing to sticky protein precipitation nor small and light droplets on to the teflon wire in the EDM module. A mixture of propan-2-ol–chloroform (25:75, v/v) has been used as extraction solvent for anticonvulsants [5] and turned out to be a good choice also for the assay of omeprazole and its metabolites.

Omeprazole and its analogues are ampholytes and uncharged between pH 6 and 8, therefore, pH 7 should be suitable for solvent extraction, cf [4]. The distribution ratios for omeprazole (**I**), the metabolites and some related compounds at this pH are shown in Table 1. Using a phase volume ratio of 1, the theoretical extraction recovery is >99% for all compounds except for **II**. Whilst batch extraction of omeprazole from plasma samples is rapid, extraction in polypropylene coils was found to be delayed. In Fig. 3 the relative recovery of omeprazole (**I**) is presented as a function of the extraction time. The extraction times were varied from 3 to 6 min by use of coils with three different lengths. Longer coils were avoided, in order to minimize risks of clogging and adsorption losses. Coils made from glass were also tested but gave no advantage compared with the polypropylene coils.

Table 1
Distribution ratios (D) for omeprazole, metabolites and the internal standard between propan-2-ol–chloroform (25:75, v/v) and phosphate buffer solutions. pH 7.0. $I = 0.10$

Compound	D
Omeprazole (**I**)	850
Hydroxyomeprazole (**II**)	18
Sulphone (**III**)	680
Sulphide (**IV**)	4700
Internal standard (**V**)	320

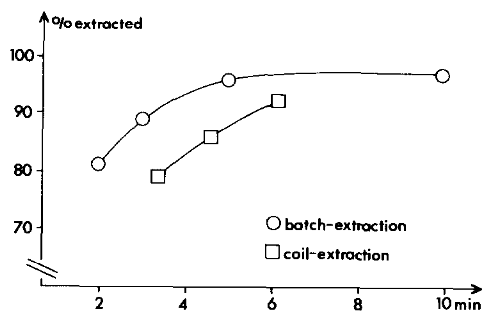

Figure 3
The relative extraction recovery for omeprazole (**I**) in plasma as a function of the extraction time. Coil-extraction and batch-extraction are compared.

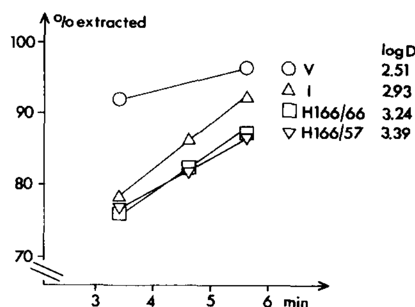

Figure 4
The relative recovery for the coil-extraction of omeprazole (**I**), internal standard (**V**) and two analogues (H 166/66, H 166/57) as a function of the extraction time.

Three analogues of omeprazole, of interest as potential internal standards, showed time-dependent extraction recovery inversely related to the distribution ratio (Fig. 4). Compound **V**, which finally was selected as internal standard, has the lowest distribution ratio but is the most rapidly extracted and was best suited to compensate for incomplete extraction, non-stoichiometry and minor experimental variations. In our method we have chosen a coil length of 4.5 m, giving an extraction time of 4–5 min.

Recovery

The absolute recoveries of the compounds are difficult to estimate because of non-stoichiometric operations during the sample preparation [6]. The acidflex pump tubes are not volumetrically tested and the flow-rate changes with time. The aliquot of organic phase utilized after the phase separation therefore varies. Losses of droplets from the teflon wire in the EDM and difficulties in capturing the whole sample plug in the loop are other factors that may influence the recovery. However, the absolute extraction recovery is at least 85% for **I**–**V** except for **IV** (sulphide), which is the most lipophilic compound of the series and which is slowly extracted.

Recoveries relative to the internal standard also were estimated. Plasma and urine samples containing **I**–**IV** were analysed and the peak height ratios relative to the internal standard were compared with the ratios obtained for a reference solution injected directly onto the column. High recoveries (95–105%) were obtained except for the sulphide (**IV**), which had only a recovery of 40% from plasma. These results suggest that the internal standard compensates well for non-stoichiometric operations but poorly for incomplete extraction. It is possible that the recovery of **IV** may be increased by a longer extraction time; however, it was decided not to determine this compound as the actual concentration in plasma is very low.

Chromatography

The reversed-phase separation of omeprazole (**I**) was performed at pH 7.7, where it was well separated from both the sulphone metabolite (**IV**) and the chosen internal standard (**V**). As can be seen in Fig. 5, no interfering peaks are observed in the chromatogram of a plasma blank close to the retention time of omeprazole. Under the conditions used the columns exhibited good stability and more than 500 samples could be processed with only a minor loss in the column efficiency. Each day, after the last run, the columns were automatically washed with acetonitrile and water, a fact that probably contributed to the good column stability. The number of theoretical plates obtained for the omeprazole peak is high at about 4000, despite the injection of volumes as large as 1.2 ml. This is due to a concentration effect at the top of the column associated with injection of the sample in an acetonitrile lean mobile phase.

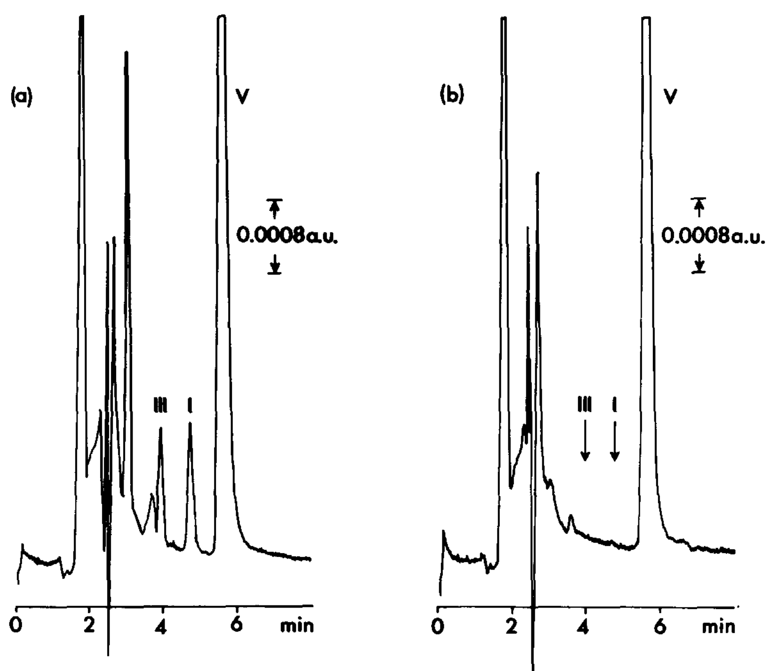

Figure 5
Chromatogram of a plasma sample after administration of omeprazole (a) and before administration (b). Isocratic elution. The concentration of omeprazole (**I**) is 260 nmol l^{-1} and that of the sulphone (**III**) 200 nmol l^{-1}.

The more hydrophilic metabolite (**II**) is not retained in the above isocratic system; however, by use of a gradient elution system it can be determined in the same run as **I** and **III** without loss of sensitivity. The selected gradient profile gives a good separation of **II** and maintained separation of the other compounds (Fig. 6). The third metabolite, the sulphide (**IV**), elutes after 13 min in this system but, as mentioned previously, it is of less interest as it is not present in patient plasma in measurable concentrations.

Certain plasma samples contained a potential metabolite, which eluted close behind **II** and was often not completely separated (Fig. 6a). However, in all assays so far

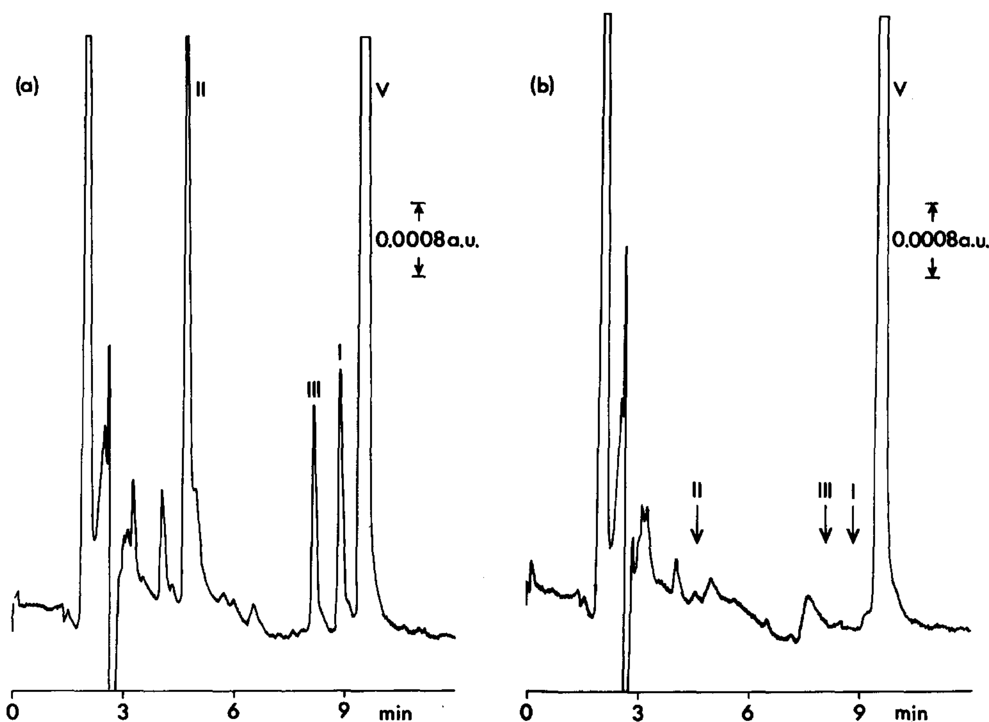

Figure 6
Chromatogram of a plasma sample after administration of omeprazole (a) and before administration (b). Gradient elution. The concentration of omeprazole (**I**) is 330 nmol l^{-1}, of hydroxyomeprazole (**II**) 1100 nmol l^{-1} and that of the sulphone (**III**) 265 nmol l^{-1}.

performed it was found to be insignificant compared to **II** and could be disregarded. In the present study no or very low concentrations of metabolite **III** are found in the urine samples. In a few samples a compound eluted close to **III**, but could be separated by increasing the mobile phase pH to 8.0. A chromatogram of an authentic urine sample is shown in Fig. 7.

Precision and accuracy

Omeprazole and its metabolites can be measured in plasma and urine samples with good precision, as shown by the repeatability and reproducibility data presented in Tables 2 and 3. Even for the sulphide (**IV**) the relative standard deviation is low despite its low extraction recovery. The repeatability was determined by replicate analysis of ten identical samples within a day. The reproducibility of the method was examined by assaying identical samples over a period of 3 months.

In plasma the limit of determination, defined as the concentration where the standard deviation is 10–15%, is 50 nmol l^{-1} for **I**, **II** and **III**. In the case of the urine method, where only 200 μl of sample is used, concentrations down to 200 nmol l^{-1} can be determined.

The linearity ranges from 0.05 to 50 μmol l^{-1} of plasma and 0.2 to 200 μmol l^{-1} of urine. The carry-over in the method was estimated to be less than 0.2% by running a reference sample with a high concentration of **I**, followed by a blank.

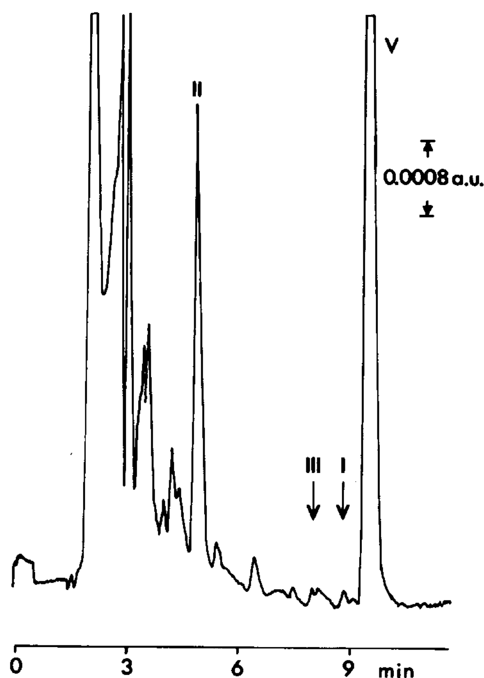

Figure 7
Chromatogram of a urine sample after administration of omeprazole. Gradient elution. The concentration of hydroxyomeprazole (**II**) is 3940 nmol l^{-1}. The excreted amounts of omeprazole (**I**) and the sulphone (**III**) in urine are very low.

Table 2
Repeatability of the determination of omeprazole (**I**) and metabolites (**II**, **III** and **IV**) in plasma and urine

	Concentration (μmol l^{-1})	RSD (%) $n = 10$ I	II	III	IV
Plasma	8.0	1.6	2.5	3.8	3.5
	0.30	4.5	4.4	5.5	11.1
Urine	16.0	1.4	1.6	1.3	—
	0.80	2.0	1.7	5.1	—

Table 3
Reproducibility of the determination of omeprazole (**I**) and metabolites (**II** and **III**) in plasma and urine

	Concentration (μmol l^{-1})	RSD (%) I	II	III	n
Plasma	2.0	2.5	6.5	3.5	72
Urine	8.0	2.1	3.5	2.2	40

The reliability of the method was evaluated against a normal-phase LC method with manual sample preparation [4]. The mean quotient of the results from the two methods was around 1.00 and the relative standard deviation was 7–9%. Both analytical methods have been used for assaying several thousands of samples. The FAST®-LC system is more complicated to set up than the conventional LC system. The initial work is, however, worthwhile as the sample capacity is higher. The precision is comparable and the limit of determination, although somewhat higher, is sufficient both for therapeutic levels and pharmacokinetic studies.

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