High-throughput solid-phase extraction for the determination of cimetidine in human plasma

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Abstract

For the implementation and validation of an automated 'high-throughput' solid-phase extraction (SPE) system, using microtiter solid-phase technology and a pipetting robot, a SPE method previously validated manually for cimetidine in human plasma was adapted. Sample cleanup was performed by means of SPE using Microlute extraction plates in the 96-well format, each well filled with 50 mg of Varian C_{18} sorbent. Separation was performed by reversed-phase high-performance liquid chromatography (HPLC) with UV detection at 234 nm. The validated calibration range was from 0.100 to 5.00 mg/l, with an inaccuracy and imprecision below 20% at all concentration levels. Validation results on linearity, specificity, precision, accuracy and stability are shown and are found to be adequate. Cross-check analysis of samples from a clinical trial showed that there is a good correlation between results obtained by the automated method and results obtained by the manual method. The average sample preparation time for a technician decreased from approximately 4 min per sample to 0.6 min. A sample throughput of at least 160 samples per day can be achieved, the HPLC analysis time being the rate-limiting step. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

The strategy of the pharmaceutical industry is to reduce the time needed for research and development of a new drug. In the screening of new potential drugs, this has led to the introduction of high-throughput techniques such as combinatorial chemistry. Consequently, more compounds become available for further investigation in a certain time period, which creates a higher workload in the following stages in the R&D process, including the workload of analytical laboratories involved in the analysis of biological samples from clinical studies.

The performance of an analytical laboratory is not only judged on the basis of the quality of the results but also on the speed of delivery. Therefore, in analytical laboratories also, there is a need for high-throughput technology. Since the introduction of liquid chromatography with tandem mass spectrometric detection (LC–MS–MS) as a quantitative technique in the field of routine bioanalysis, a new bottleneck in the total analytical process appeared. The analysis time on the analytical instrument was no longer the limiting step. Instead, the sample preparation became the rate-limiting step. In the last

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decades, only little progress was made in the automation of sample preparation, in spite of the fact that surveys showed that the staff in analytical laboratories spend more than 60% of their time on sample preparation [1].

Since its introduction in the mid-eighties, solid-phase extraction (SPE) has become one of the most popular sample preparation techniques for the analysis of drugs in biological fluids. SPE generally gives higher recoveries, is more reproducible, is less time-consuming and is easier to automate compared to the traditional liquid extraction methods [2].

Recently, Kaye et al. [3] introduced a novel method for efficient SPE. They developed techniques for SPE based on 96 well plates, which are commonly used in biochemical analysis. The 96 wells SPE technology allows for high-throughput SPE by processing 96 samples in a standard 8×12 microtiter plate format. Sample processing is performed 'off-line' and in 'batchmode' and is therefore ideal for LC–MS–MS and other high-throughput applications [3–5].

We have implemented instrumentation for the automated 96 wells SPE. To validate the system thus installed, and to evaluate its potential advantages with regard to increased throughput of bioanalytical samples with equal human involvement (hence decreasing costs per sample and reporting times), we have adapted a validated manual SPE method for cimetidine in human plasma (unpublished data). Another goal of this work was to inventorize the potential problems and drawbacks of such a system in routine applications.

The sample preparation of cimetidine (see Fig. 1), a specific histamine H_2-receptor antagonist that inhibits hypersecretion of gastric acid from parietal cells in the gastric mucosa [6], was automated and

the new method thus obtained was validated and compared with the manual method.

2. Experimental

2.1. Chemicals and reagents

Cimetidine and the internal standard, ornidazole, were supplied by Sigma (St. Louis, MO, USA). Acetonitrile, methanol and phosphoric acid were of HPLC grade and supplied by J.T. Baker (Philipsburg, NJ, USA). Acetic acid, ammonium acetate, diethylamine disodium hydrogen phosphate dihydrate and hydrochloric acid were all of analytical grade and supplied by Merck (Darmstadt, Germany). Water was purified using a Milli-Ro-10 and a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Solid-phase extraction system

The SPE system consisted of a Canberra Packard Multiprobe 104 pipetting robot (Canberra Packard Instruments, Dowers Grove, CT, USA) with 1.0 ml syringes, a vacuum pump (Edwards International, Crawley, UK) and a solid-phase extraction system version 2 (Canberra Packard UK) or version 3 (Canberra Packard Benelux). In version 2 (used during the implementation and validation of the method) of the SPE system, two two-way valves were used to control a pulsed vacuum. In version 3 (used during the cross-check analysis), one three-way valve was placed between the wash bottle and the vacuum manifold. This results in a better control over the flow-rates of sample and wash- or elution solvents. Moreover, a higher vacuum can be achieved, resulting in a reduced incidence of blockage of the extraction columns. A vacuum control diagram is given in Fig. 2.

Extractions were performed on a Microlute™ vacuum manifold (Porvair Sciences, Shepperton, UK) and Microlute 96 well extraction plates with 1.5 ml volume extensions, with each well filled with 50 mg of Varian C_{18} SPE sorbent (also supplied by Porvair). Samples were collected in Porvair deep-well collection plates with a round-bottomed well profile and a capacity of 1 ml.
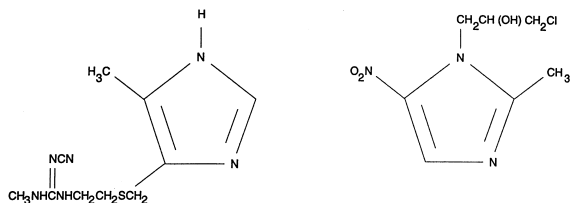

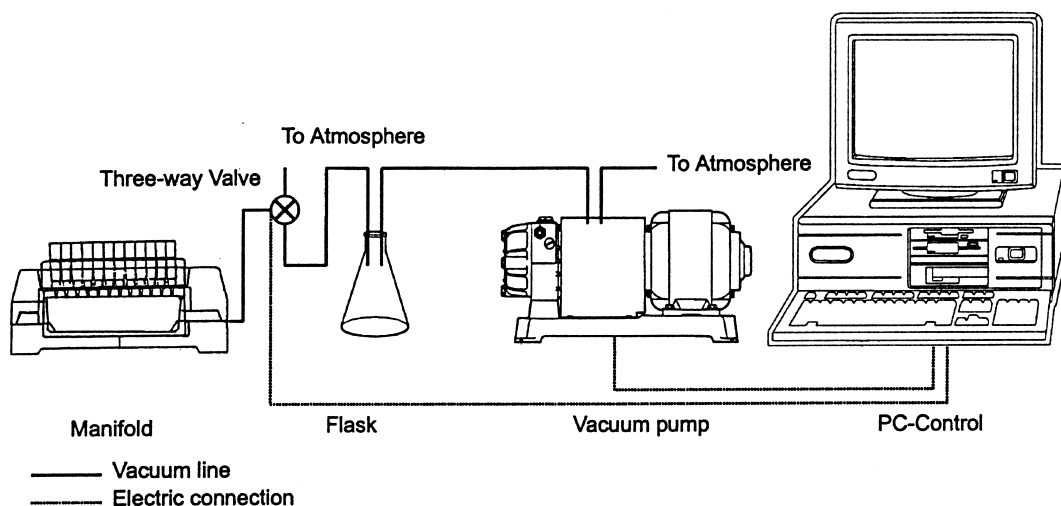Fig. 1. Structural formulae of cimetidine and ornidazole.

Fig. 2. Vacuum control diagram of version 3 of the solid-phase extraction system.

2.3. Chromatographic system

A Waters (Milford, MA, USA) Model M 616 system delivered the mobile phase (a mixture containing 2175 ml of 0.1 M acetate buffer (pH 7.0) 275 ml of acetonitrile and 50 ml of methanol) at a flow-rate of 1.0 ml/min. Injections of 40 µl were made using a Waters Autosampler Model 717 plus, equipped with a cooling module set at a temperature of 10°C. Reversed-phase high-performance liquid chromatography was performed using a Chrompack (Middelburg, The Netherlands) 3 µm Microsphere C_{18} cartridge column (100×4.6 mm) with a Chrompack guard column type R2 (10×2.0 mm) conditioned at a temperature of 40°C by means of a W.O. Electronics (Langenzersdorf, Germany) BEO-04-fl column thermostatic oven. Detection was performed with a Kratos (Ramsey, NJ, USA) Spectroflow 783 detector at a wavelength of 234 nm using a range of 0.002 AUFS and a risetime of 2 s. The Waters Millennium 2020 version 2.15 Chromatography Manager was used for data analysis.

2.4. Preparation of calibration and validation samples

Stock solutions of cimetidine were prepared by dissolving 50.0 mg of cimetidine in 50.0 ml of 0.001 M hydrochloric acid. Calibration samples and validation samples were prepared by properly diluting the stock solutions of cimetidine with blank, analyte-free, human plasma. For calibration, eight plasma pools were prepared, containing 0.100, 0.250, 0.500, 1.00, 1.50, 2.00, 3.00 and 5.00 mg/l. For the validation samples, four additional pools were prepared, from an independent stock solution, containing cimetidine concentrations of 0.100, 0.200, 2.00 and 4.00 mg/l. For quality control during the cross-check analysis, another four additional pools were prepared. These quality control pools had cimetidine concentrations of 0.100, 0.250, 1.00 and 4.00 mg/l.

2.5. Internal standard solution

A stock solution of ornidazole was prepared by dissolving 50.0 mg of ornidazole in 50.0 ml of 0.1 M phosphate buffer (pH 7.0). This stock solution was diluted with water to prepare a working solution with a concentration of 20.0 mg/l.

2.6. Sample preparation

Within 2 h prior to analysis, all samples, including calibration and validation samples, were thawed in a waterbath at 30°C for 15 min. Subsequently, the samples were homogenised and centrifuged for ten

min at 3500 g prior to extraction on the Microlute extraction column using the modified Canberra Packard pipetting robot. Following conditioning with 1.0 ml of methanol, 1.0 ml of water and 1.0 ml of 0.1 M phosphate buffer (pH 7.0), the Multiprobe sequentially aspirated 50 µl of internal standard, 150 µl of 0.1 M phosphate buffer (pH 7.0) and 250 µl of plasma sample. These aliquots were dispensed to the individual wells of the extraction plate at a high speed, providing adequate mixing. After washing with 500 µl of 0.1 M phosphate buffer (pH 7.0) and 1000 µl of water, the samples were eluted into a deep well collection plate by three subsequent elution steps with 100 µl of methanol, 150 µl of methanol and 250 µl of 0.01 M acetate buffer (pH 7.0). The extracts were transferred to vials and placed in the autosampler. Injection volumes of 40 µl were used in the HPLC system. A detailed overview of the sample preparation procedure is given in Table 1.

2.7. Validation experiments

The automated method was validated over the range from 0.100 to 5.00 mg/l using the validation approach of Wieling et al. [7] with small modifications.

The *linearity* of the method was assessed by measuring the peak height ratios of cimetidine/internal standard versus the concentration (weighting factor $1/X$) in eight calibration samples, in triplicate, and performing a goodness of fit and lack of fit test by analysis of variance.

The *selectivity* of the assay was assessed by analysing blank plasma samples from six different healthy individuals, standard solutions of the pure compounds and samples from subjects after administration of a dose of cimetidine.

The *accuracy and precision* at concentration levels of 0.100 (LOQ), 0.200, 2.00 and 4.00 mg/l were determined by analysing the validation samples in sixfold during six analytical runs. The overall within-run and between-run precision was calculated using one-way ANOVA.

The *recovery* was determined at three concentration levels (0.200, 2.00 and 4.00 mg/l) by comparing the peak height of the extracted precision and accuracy samples ($n=6$) with the peak height of non-extracted standard solutions of the corresponding concentration ($n=6$) in six analytical runs.

For the assessment of the *autosampler stability*, pooled extracts of spiked plasma samples at two concentrations (0.200 and 4.00 mg/l) were injected every 2 h for a total period of 30 h, during which time, the extracts were kept in the sample compartment of the injector (protected from light and at a temperature of 10°C). The peak height ratios of cimetidine/internal standard were evaluated using regression analysis.

The stability after repeated freezing and thawing was already proven during the validation of the manual method (described in an internal report). The experiments were not repeated during validation of the automated method.

Dilution of samples of cimetidine was investigated by analysing over-curve control samples; one con-

Table 1
Overview of the sample preparation procedure

Step	Process	Reagent	Volume (µl)	Vacuum (s)
1	Conditioning	Methanol	1000	12
2		Water	1000	20
3		Phosphate buffer	1000	20
4	Sample loading	Internal standard	50	90
		Phosphate buffer	150	
		Plasma sample	250	
5	Washing	Phosphate buffer	500	45
6		Water	1000	45
7	Elution	Methanol	100	6
8		Methanol	150	6
9		Acetate buffer	250	10

centration (8.00 mg/l) was diluted four times in sixfold, to obtain a nominal diluted concentration of 2.00 mg/l in four analytical runs. The data were evaluated comparing the precision and accuracy obtained after analysis of variance with the data from the precision and accuracy experiments at a concentration of 2.00 mg/l.

2.8. Cross-check analysis

The performance of the method applied to clinical study samples was studied by performing a cross-check analysis. A batch of 48 samples from the clinical study in which subjects received a dose of 400 mg of cimetidine were analysed with the automated method in one analytical run. These samples had been analysed previously using the manual method. Data were evaluated by regression analysis and by calculation of the absolute relative difference.

3. Results and discussion

3.1. Performance of the method

The sample preparation procedure from the manual method was slightly modified. In the original method, Baker C_{18} 100 mg cartridges were used. For the automated method, this was changed to 50 mg of sorbent per well. Also, the required sample volume was reduced from 500 to 250 μl and the wash and elution volumes were reduced by a factor of two, resulting in the same concentration of the final extract. Reducing reagent volumes provides for faster sample processing because of a more efficient use of the multipipetting facility of the pipetting robot and less chance of blocked columns.

The time needed for transfer of a manual method to the 96 well format mostly depends on whether or not the same chemistry can be used in the manual and the automated method. In our experience, programming and testing of the vacuum settings of the automated system takes approximately two days. Time needed for testing and adjustment of these settings depends on the robustness of the SPE procedure. For the implementation of the cimetidine assay, we needed approximately five days.

During sample preparation of the clinical samples used for cross-check analysis, one of the samples was lost, due to blockage of a column. Blocking is a well-known problem in SPE. Individual cartridges or wells become clogged after the addition of matrix. This problem, mainly caused by solid particles in the sample, can be eliminated for the most part by thorough centrifugation. Blocking may have a significant effect on the economical advantages of automated analysis because of the re-analysis that has to be done in an additional analytical run. This effect is more significant with a smaller number of samples to be analysed in a clinical study.

To prevent contamination of other wells in an extraction plate, the 'liquid sensing' facility of the Canberra Packard pipetting robot can be used. With this standard option of the robot, a program can be written that detects whether or not any liquid is left in a well after loading of the sample and the subsequent application of vacuum. If there is liquid left in a well, it will be removed and transferred to waste so that no wells close to the blocked well are contaminated during the subsequent washing and elution steps. Identification of blocked wells will be stored in a database file.

For a batch of 96 samples (including a blank, eight calibration samples and six quality control samples), a sample processing time of about 55 min was required. The sample throughput was increased from 50 samples using the manual method (limited by the manual SPE method) to 160 samples per day after automation (with calibration and quality control samples included). The limiting factor in the analytical procedure for cimetidine is the chromatographic runtime of 7.5 min (retention time of cimetidine is ≈4.5 min and of ornidazole is ≈6.8 min) and, therefore, no higher throughput than approximately 160 samples per 24 h can be achieved. The use of high-throughput analytical techniques, such as LC–MS–MS, can further increase the sample throughput.

The method presented is semi-automated and still needs human intervention at different stages in the sample preparation process. The samples have to be placed in the sample racks of the robot system; before elution, the deepwell plate has to be put into the vacuum manifold and the prepared samples have to be transferred to vials before injection. The use of XYZ autosamplers and robotic gripper arms may

yield applications that are totally unattended and can also further increase the sample throughput.

3.2. Validation results

The *linearity* of the method was established for the concentration range of 0.100 to 5.00 mg/l. A test for lack of fit showed that the first-order model ($y=ax+b$), with a weighting factor of $1/X$, is appropriate for establishing a relationship between concentration and response ($r=0.999$). The goodness of fit (F-test for regression) is highly significant [$F_{calc}=5066$, F_{Table} ($\alpha=0.05$)=4.30]. No significant lack of fit was observed [$F_{calc}=0.73$, F_{Table} ($\alpha=0.05$)=2.74], with residuals being the result of experimental error rather than a consequence of model deviations.

With respect to the *selectivity*, no major interferences (<0.5 times the response of the LLQ) were found at the retention times of cimetidine or the internal standard (see Fig. 3).

For the *lower limit of quantitation*, the lowest concentration of the calibration curve, 0.100 mg/l, was taken. The precision and accuracy at this level were within acceptable limits (C.V. and bias $\leq 20\%$).

A summary of the results of the *precision and accuracy* experiments is given in Table 2. The overall precision (C.V.%) of the method was better than 15% at all concentration levels ($n=35$). The within-run and between-run precisions (C.V.%) were better than 11.7 and 19.6%, respectively. The bias varied between -4.7 and $+4.7\%$ at all concentration levels. The results meet the criteria established during the Washington Meeting on Analytical Methods Validation [8].

The *recovery* of cimetidine was consistent over the entire calibration range and was, on average, 82%. The recovery of the manual method was 94%. For ornidazole, the recovery was determined on the concentration normally used during routine analysis (plasma concentration of 4.00 mg/l) and was found to be 72%. This is lower than the manual method, where it was 89%. Besides having comparable chromatographic behaviour, ornidazole also displays
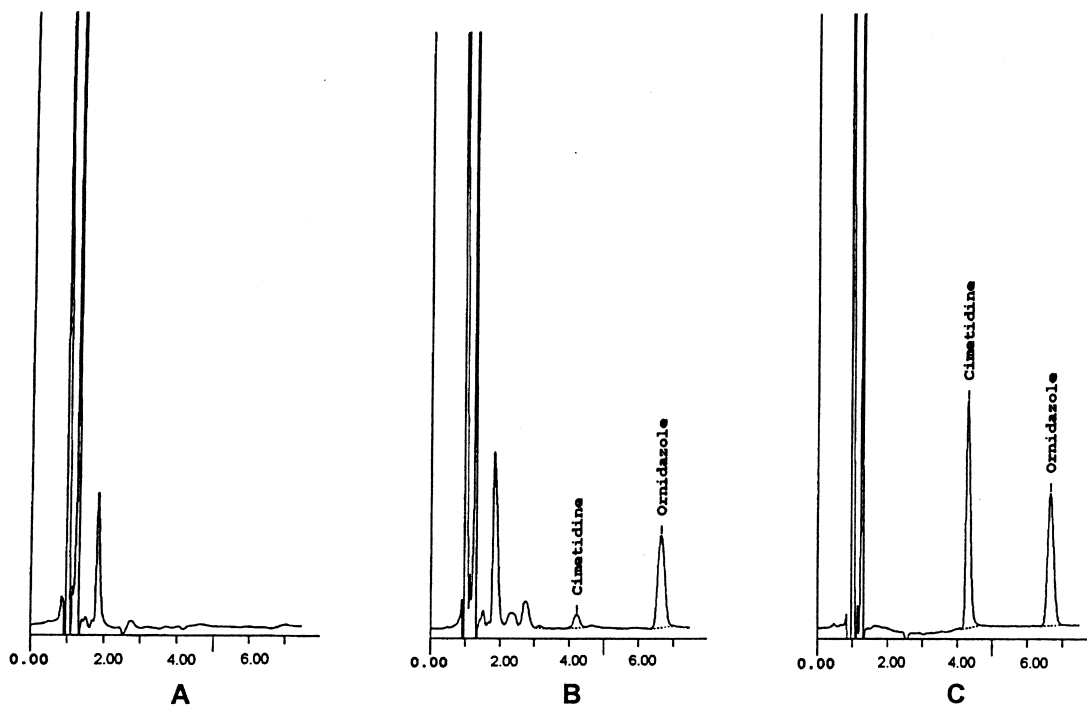

Fig. 3. Representative chromatograms. (A) Blank human plasma; (B) spiked plasma sample at the LOQ (0.100 mg/l) and (C) study sample from a subject after a dose of 400 mg of cimetidine (retention time of cimetidine ≈ 4.2 min and of ornidazole (I.S.) ≈ 6.8 min).

Table 2
Summary of the results of the validation of precision, accuracy and recovery

Nominal concentration (mg/l)	Measured concentration (mg/l)	Bias (%)	Overall C.V. (%)	Within-run C.V. (%)	Between-run C.V. (%)	Recovery (%)	n
0.100	0.105	4.7	13.1	11.6	19.5	ND	35
0.200	0.191	−4.7	9.7	8.6	14.7	84.6	36
2.00	2.00	0.2	2.1	2.2	1.7	80.9	35
4.00	3.93	−1.7	2.4	2.0	3.9	80.5	34

ND=not determined.

a similar extraction behaviour, which makes this compound a good choice as the internal standard for this assay.

The results on the *stability* of cimetidine and ornidazole in the compartment of the autosampler at 10°C show a small increase (+1.5%) in the response ratio at the concentration level of 0.200 mg/l and a decrease of 3.2% of the response at the concentration of 4.00 mg/l, indicating that there are no signs of deterioration under these storage conditions.

The results obtained during the *dilution* experiments are comparable with the data obtained during the precision and accuracy experiments. The within-run and between-run precisions were 2.2 and 2.2%, respectively, after dilution. The bias was 0.9%. The data show that partial volume analysis is allowed in cases where the concentrations of the samples being studied are above the calibration curve values or when there is insufficient sample to perform an assay.

3.3. Cross-check analysis

The results of the cross-check analysis are also given and are graphically presented in Fig. 4. They show an indication that there is a small proportional difference between the automated and the manual results (slope=0.949±0.023). However, the quality control data obtained during that analytical run show the same inaccuracy as the cross-check results (see Table 3). There is no indication for a systematic error (the intercept value of 0.0099±0.0615 does not deviate significantly from zero). The correlation between the two sets of data is good ($r=0.994$). The mean relative difference between the manual results and the results of the automated method was 5.5% ($n=46$), which is good compared to the coefficient of

variation found for precision and accuracy during validation. There is no significant effect of concentration on the relative difference between the manual results and the automated results.

3.4. Application of the method

Considering the number of samples that can be analysed per day and the quality characteristics, the automated method is suitable for use in the routine analysis of plasma samples in pharmacokinetic studies. An example of a concentration–time curve from a subject who participated in a clinical study in which subjects received a dose containing 400 mg of cimetidine is given in Fig. 5.

4. Conclusions

An automated 'high-throughput' sample preparation procedure for cimetidine in plasma using mi-
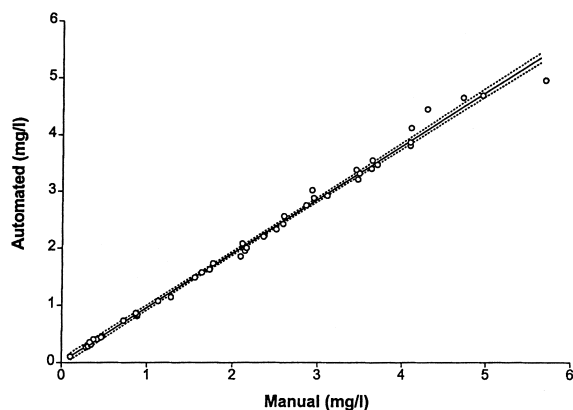

Fig. 4. Cross-check analysis. Evaluation by regression analysis of the automated results vs. the manual results.

Table 3
Quality control results obtained during the cross-check analysis

Nominal concentration (mg/l)	Observed concentration (mg/l)	Bias (%)	SD (mg/l)	C.V. (%)	n
0.100	0.095	−4.8	0.017	17.6	6
0.250	0.223	−10.8	0.048	2.2	6
1.00	0.904	−9.6	0.015	1.6	6
4.00	3.80	−5.1	0.059	1.5	6

crotiter plate solid-phase extraction technology was implemented successfully. Validation results show that the sample throughput can be increased by at least a factor of three without a loss of quality of the analytical results. The automated method shows good accuracy and acceptable precision at all concentration levels. There is also a good correlation between the results obtained by the automated method and the results obtained by the manual method. Suggestions for further automation were discussed.
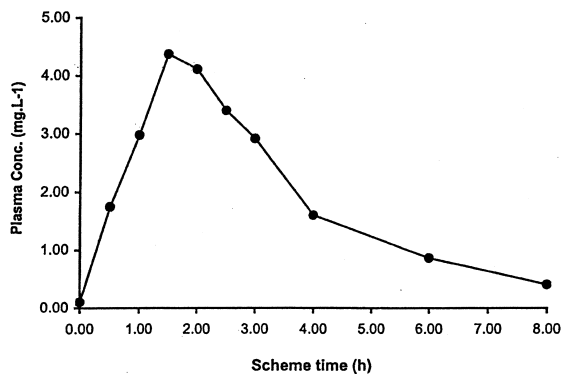

Fig. 5. Plasma concentration–time curve. Application of the method in a clinical study using cimetidine.

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