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Development of a laboratory robotic system for automated bioanalytical methods — I. The determination of theophylline in human plasma: a comparison between the robotized and manual method

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Abstract: The quality of bioanalytical methods is often determined by the quality of sample preparation. Using a robot for sample treatment may give better results than manual sample preparation, since the robot lacks human behaviour and incidental errors that are part of it. The use of a laboratory robot has the additional advantage of giving each sample the same analytical history, resulting in better reproducibility.

An automated method has been developed for the analysis of drugs in plasma using a laboratory robot. Theophylline was used as a probe drug. Sample preparation was automated with a Zymate II robot, followed by separation and quantitation on an HPLC-system. The robotic method showed a good correlation with the manual method, while sample throughput was doubled.

Keywords: *Robotic sample preparation; robot control program; theophylline in plasma; validation of robotic method; comparison with manual sample preparation.*

Introduction

Complex sample preparation is often required in order to make samples suitable for HPLC-analysis. Drugs to be determined have to be extracted from the biological matrix in a selective and quantitative manner. The quality of the sample pre-treatment determines the performance of the analytical method. Over the last few years, many different types of automated sample preparation techniques have been developed. Turnell and Cooper [1] have described an automated system combining dialysis and trace enrichment. The use of automated solid-phase extraction on line with an HPLC-system is another popular technique.

Automation of the sample preparation is also possible by using a laboratory robot. Moreover, the robot can inject the pre-treated sample directly into an HPLC-system, making on-line analysis possible.

Several papers discussing laboratory robots have already been published since the first appearance of these robots in 1982. Strimaitis and Hawk [2] have shown the use of robotic systems in pharmaceutical studies, material sciences and high technology. Hawk *et al.* [3] described a disciplined approach to sample preparation and a robotic system for automation of sample preparation as part of an integrated analytical method. Hayashi *et al.* [4] and Matsuda *et al.* [5] have used a laboratory robot for the total analysis of solid dosage formulations. Schmidt *et al.* [6] have described a procedure utilizing laboratory robotics for performing precolumn derivatization prior to liquid chromatographic analysis. Laws and Jones [7] used a laboratory robot for the flexible automation of pesticide residue analysis. Strimaitis [8] has discussed the advantages of a laboratory robot in the analytical laboratory according to productivity, method

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development and safety. Fouda and Schneider [9] have reported the integration of a Zymate robot and components into a flexible system for the automated analysis of drugs in biological fluids.

A robot is eminently suitable for performing a number of actions with high precision. These actions are coordinated by means of a robot computer, the controller. Detailed information on robotic hardware has been reported in a number of articles [10–15].

The main advantages of a robot over other automated methods are its flexibility, its programming abilities and the possibility of linking it with other kinds of laboratory apparatus also controlled by the controller. It may improve the overall efficiency of the laboratory.

This paper describes the fully automated sample preparation for the analysis of theophylline in human plasma. The aim was to gain experience with a laboratory robotic system, to evaluate the robotic procedure and to compare its accuracy and performance with the manual procedure.

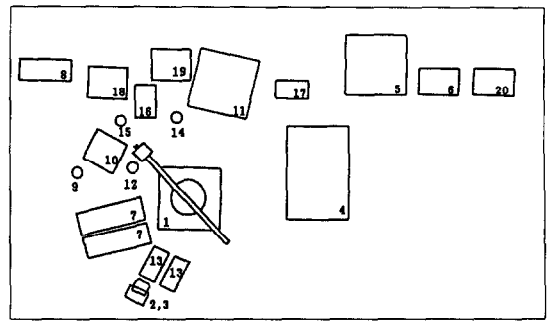

Figure 1
Bench-layout of the robotic system. (1) robot with arm; (2) dual function hand; (3) parking-station for dual function hand; (4) controller; (5) keyboard, monitor and diskdrive; (6) printer; (7) racks for 10 ml tubes; (8) master lab station; (9) dispensing nozzle; (10) vortex mixer; (11) centrifuge; (12) dummy station; (13) 1 ml pipettip racks; (14) sample waste; (15) pipettip waste; (16) HPLC injection station; (17) analytical instrument interface; (18) HPLC-pump; (19) HPLC-detector; (20) integrator.

Experimental

Apparatus

A commercially available Zymate II robot system (Zymark Corporation, Inc., Hopkinton, MA, USA) was used. The main parts of the robotic system were the controller (the robot control computer), the laboratory stations and the robot arm (used for moving sample tubes from station to station). Laboratory stations were the vortex mixer, the centrifuge and the Master Lab Station (MLS) which dispensed reagents into sample tubes. These stations were switched on and off by the robot control computer which also directed the robotic arm. Figure 1 shows a bench-layout of the total robotic system. The system consisted of a Zymate Z 110 robotic arm, a Zymark Z 905 dual function hand, a Zymark Z 510 Master Lab Station, a Zymark Z 620 Single tube vortexing unit, a Zymark Z 710 centrifuge and a Zymark Z 310 HPLC-injection station equipped with an electrically controlled Rheodyne valve. The Zymark Z 310 Analytical Instrument Interface was used to control the HPLC-injection station.

The chromatographic apparatus consisted of a Waters Model 45 HPLC-pump, equipped with a Waters Model 441 fixed wavelength UV-detector (280 nm) and a Spectra Physics SP4270 computing integrator. For the manual procedure a Waters 712 WISP autosampler was used.

Chemicals

Theophylline (1,3-dimethylxanthine) and the internal standard (β-hydroxyethyltheophylline) were obtained from Sigma Chemical Company (St. Louis, MO 63178, USA), ammonium sulphate was supplied by Merck (D-6100, Darmstadt, FRG), chloroform Chrom QR by Promochem (Promochem GmbH, D-4230, Wesel, FRG) and isopropanol by Fisons (Fisons plc, Scientific Equipment Division, Loughborough, LE 11, ORG, UK). Water was purified by using a Milli-RO-4 and a Milli-Q water purification system (Millipore Corp., Bedford, MA 01730, USA). Newborn bovine serum was obtained from Flow Laboratories Ltd (Irvine, KL2 8NB, UK).

Choice of assay method

The analysis of theophylline in plasma was chosen as a model for the validation of sample preparation in bioanalytical methods executed by a laboratory robot. This method complies with the following requirements:

— the existing non-automated method has proved to be robust and its performance is well known;
— the method is relatively simple; the number of handling stations can be limited and validation does not depend on a large number of components;

— samples are sufficiently stable;
— the laboratory has much experience with the method [16–18].

Manual procedure

The manual sample preparation for the analysis of theophylline in plasma was carried out as follows: 200 μl of plasma were pipetted into a 5 ml polypropylene tube; 200 μl of water, 200 μl of internal standard solution and 200 μl of a saturated ammonium sulphate solution were added, and the mixture was vortexed for 10 s. 1.6 ml of a chloroform–isopropanol (95:5) mixture was added and vortexing continued for a further 80 s. The tube was centrifuged for 10 min at 5000 rpm. The organic layer was moved into autosampler vials. Next 20 μl were injected by autosampler onto a Lichrosorb Si-60 column (Merck, D-6100 Darmstadt, FRG). The mobile phase consisted of a mixture of chloroform (1325 ml), n-heptane (950 ml), methanol (225 ml), tetrahydrofuran (12.5 ml) and acetic acid (1.25 ml). For calibration, 200 μl of blank plasma was used and water was substituted by 200 μl of a calibration solution.

Robotic procedure

The aim was to automate completely the sample pre-treatment, with the exception of pipetting the 200 μl plasma sample in polyethylene tubes. This has to be done by a technician.

An addition to the manual sample preparation is the on-line injection of the pre-treated samples directly into the HPLC-system by the robot. This had the advantage of on-line data processing: immediately after the pre-treatment of a sample, the extract is injected into the system. The data of the chromatogram then can be used for guarding the robotic sample preparation and for guarding the chromatographic system. This will be discussed in Part II of this paper. Another advantage of on-line injection is that no autosampler is needed.

The automated method was built up from a number of "Laboratory Unit Operations" (LUOs): (1) manipulation by the robot arm, (2) liquid handling by dispensing/pipetting stations, (3) conditioning by a vortex mixer and (4) separation by a centrifuge.

All stations used by the robotic system (vortex mixer, centrifuge and liquid handling stations) were controlled by the robot controller.

For security reasons a few modifications were made to the manual method. Firstly, 10 ml tubes were used instead of 5 ml tubes. This saved the use of one component (a capping station) and a pre-treatment step, because these large tubes do not have to be capped before vortexing or centrifugation. Secondly, the samples were centrifuged twice instead of once; after one centrifugation step the organic layer and the aqueous layer were not separated properly in some cases. A robotic system cannot detect improper separations. Injection of an emulsion into an HPLC-system would cause problems, avoided by centrifuging twice, with a second short vortex mixing step in between. Thirdly, calibration was performed by using spiked plasma. Fourthly, the protein precipitation solution (ammonium sulphate) and the internal standard solution were added simultaneously by the Master Lab Station (MLS). Lastly, the organic layer (the lower layer in the tube) was pipetted from the tube by the syringe hand and injected directly into the HPLC-system.

The vortex speed was determined experimentally by judging the mixing process visually. Proper mixing was achieved at a vortex speed of 80 rpm. Even better mixing was obtained with a pulsating shake (alternately 10 s at vortex speed 80 and 2 s at vortex speed 0).

Developing a one-sample program

Before developing a program for automatic sample preparation of a large batch of samples, the automation of one sample has to be performed. Next, the one-sample program can be adjusted and serialized for the simultaneous handling of several samples.

When developing a one-sample program the procedure has to be divided into Laboratory Unit Operations (LUO). Figure 2 shows some examples of LUOs which were used to write the program by which the robot control computer could control the pre-treatment of one sample.

Serializing the one-sample program

Once a one-sample program has been developed, sample preparation has to be serialized. The aim of this is to give each sample an identical "history" and to use robot time as

GOAL	STEPS	LUO
Add internal standard	Move sample to dispenser	Manipulation
	Dispense internal standard	Liquid handling
Vortex	Put sample in vortexer	Manipulation
	Vortex	Condition
	Take sample from vortexer	Manipulation

Figure 2
Example of laboratory unit operation (LUO) development. Manipulation, movement of tube by robotic arm; condition, preparation of a sample in a laboratory station; liquid handling, dispensing of reagents to sample.

efficiently as possible. This can be obtained by processing more samples concurrently.

Serializing was done as follows: firstly, the one-sample program was divided into a number of sub-programs. One complete sample treatment step, e.g. centrifuging, was performed by a sub-program. Then each sub-program was timed and the rate limiting element (RLE) was determined. In the case of the determination of theophylline in plasma, the RLE was the chromatography time. Thirdly, control programs were defined, controlling one functional operation consisting of several sub-programs. The help of timers ensured identical sample histories. One timer was dependent on the RLE and determined the sample throughput. Each 7.6 min a sample was injected into the HPLC-system (chromatography time 7.5 min). The resulting serialized program was twice as long as the one-sample program (25 kB versus 12.5 kB). This is an indication of the complexity of serializing.

Modification of dispensing nozzle characteristics

Crucial for a good performance of the method is the performance of the Master Lab Station (MLS). This is the component that dispenses the precipitation solution, the internal standard solution and the extraction liquid. The precision of the dispensed volume of internal standard is of particular importance.

The characteristics of the dispensing nozzle (the point where the MLS dispenses the liquids into the tubes) determine the MLS-performance. Using the nozzle as delivered by the supplier, it was only possible to position tubes perpendicularly under the nozzle. This configuration had an important disadvantage with grave analytical consequences since drops of 10–20 µl remained at the outlets. Positioning the tubes at an angle below the nozzle and allowing all outlets to touch the inside of the tube gave better results (RSD = 1%; n = 7).

An even better configuration was obtained, by fixing the end of pipettips to the nozzles (RSD = 0.2%; n = 7, comparable to manual precision with an Eppendorf Multipette (RSD = 0.25%; n = 7)). Pressurized air was used to blow off remaining drops. Figure 3 shows the reconstructed nozzle.

Results and Discussion

Before comparing the robotic procedure with the manual procedure, the performance of the hardware was validated. As mentioned above, a few adjustments were made to the configuration of the dispensing nozzle of the MLS. The internal standard syringe showed a bias deviation of −3%. The accuracy of the dispensed volume of internal standard solution is important for the quality of HPLC-analyses, and it was found necessary to increase it by a factor of 1.03. The volumes of ammonium sulphate and extraction liquid are less important. The MLS is validated with water and with the liquids necessary for the theophylline assay by weighing polypropylene tubes before and after dispensing. Table 1 gives the results of the validation.

Results are comparable with the manual procedure. The applied modifications gave in a better performance than claimed by the supplier (RSD = 1%; accuracy of 0.5% at a volume of 20% of the syringe-volume).

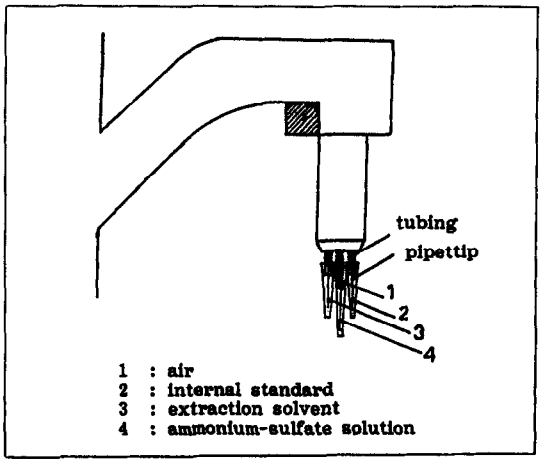

Figure 3
Modified dispensing nozzle. Nozzles are placed at different heights, pipettips are placed at the outlets.

Table 1
Precision and accuracy of the Master Lab Station

Syringe	Volume	Wanted volume	Observed volume	RSD (n = 7)	Solution
A	1.0 ml	200 µl	199.8 µl	0.17%	I.S.
B	1.0 ml	200 µl	201.1 µl	0.43%	water
B	1.0 ml	200 µl	199.7 µl	0.09%	A.S.
C	5.0 ml	1.6 ml	1.56 ml	0.15%	water
C	5.0 ml	1.6 ml	1.53 ml	0.26%	E.L.

I.S. = Internal Standard; E.L. = Extraction Liquid; A.S. = Ammonium sulphate solution.

Table 2
Analytical recovery of theophylline and the internal standard using the automated method

Component	Concentration (µg ml^{-1})	Recovery (%)	SD	RSD (%)	n
Theophylline	5.0	100.5	0.65	0.7	7
Theophylline	20.0	99.1	0.36	0.4	7
Internal std.	20.0	99.5	0.71	0.7	7

Table 3
Within day reproducibility of the automated method

Theoretical concentration µ (µg ml^{-1})	Measured concentration (µg ml^{-1}) Mean	SD	RSD (%)	Mean − µ (%)	n
1.0	1.03	0.029	2.9	+3.0	32
2.0	2.00	0.037	1.9	−0.1	31
5.0	5.04	0.051	1.0	+0.8	30
10.0	9.97	0.084	0.8	−0.3	30
15.0	14.92	0.168	1.1	−0.6	31
20.0	19.94	0.245	1.2	−0.3	30

Table 4
Within day reproducibility of the manual method

Theoretical concentration µ (µg ml^{-1})	Measured concentration (µg ml^{-1}) Mean	SD	RSD (%)	Mean − µ (%)	n
1.0	0.98	0.029	2.9	−2.4	32
2.0	1.97	0.048	2.4	−1.7	32
5.0	5.03	0.069	1.4	+0.6	32
10.0	10.03	0.067	0.7	+0.3	31
15.0	15.05	0.113	0.8	+0.3	32
20.0	19.94	0.124	0.6	−0.3	32

Table 5
Between day reproducibility of the automated method

Theoretical concentration µ (µg ml^{-1})	Measured concentration (µg ml^{-1}) Mean	SD	RSD (%)	Mean − µ (%)	n
2.0	1.96	0.056	2.9	−2.0	20
12.0	11.95	0.122	1.0	−0.4	19

Table 6
Between day reproducibility of the manual method

Theoretical concentration µ (µg ml^{-1})	Measured concentration (µg ml^{-1}) Mean	SD	RSD (%)	Mean − µ (%)	n
2.0	2.01	0.056	2.8	+0.5	19
12.0	11.78	0.239	2.0	−1.8	20

The vortex mixer is responsible for the quality of the extraction. The analytical recovery from plasma was determined to quantify the performance of the mixer. The values of the recovery as obtained with the automated method were higher than after manual extraction. This was mainly due to the double vortex mixing and centrifugation step and to the fact that the robotic system vortex mixer operates with a pulsating shake. Results are given in Table 2.

The totally automated method is validated by means of the within day and between day reproducibility compared with the manual method.

Within day reproducibility tests were performed with six pools of spiked plasma. Between day reproducibility tests have been carried out with two pools of spiked plasma. Over a 10-day period each pool was analysed in duplicate. Tables 3 and 4 show the results of the within day reproducibility experiments and Tables 5 and 6 the results of the between day reproducibility experiments. In the within day tests the reproducibility was very good and comparable with the manual method.

In the between day tests, the reproducibility at a concentration of 2.0 μg ml^{-1} is comparable with the manual method. At a concentration of 12.0 μg ml^{-1}, however, the reproducibility is significantly better than the manual method (F-test; $p < 0.01$).

The described theophylline assay technique is very robust but with a less robust method the reproducibility of the robotic method may be much better than the manual method. In addition, the manual method for the determination of theophylline in plasma was carried out by a technician very experienced in this method. The accuracy, measured as the deviation of the mean of the measured concentration from the theoretical concentration, is less than the manual method at a concentration level of 2.0 μg ml^{-1} and this deviation is significant (F-test; $p < 0.005$); at a concentration level 12.0 μg ml^{-1}, however, the accuracy is better with the deviation from the theoretical value not significant.

To investigate the correlation of the automated with the manual method, a series of unknown samples, previously analysed by the manual method, was determined by the robotic

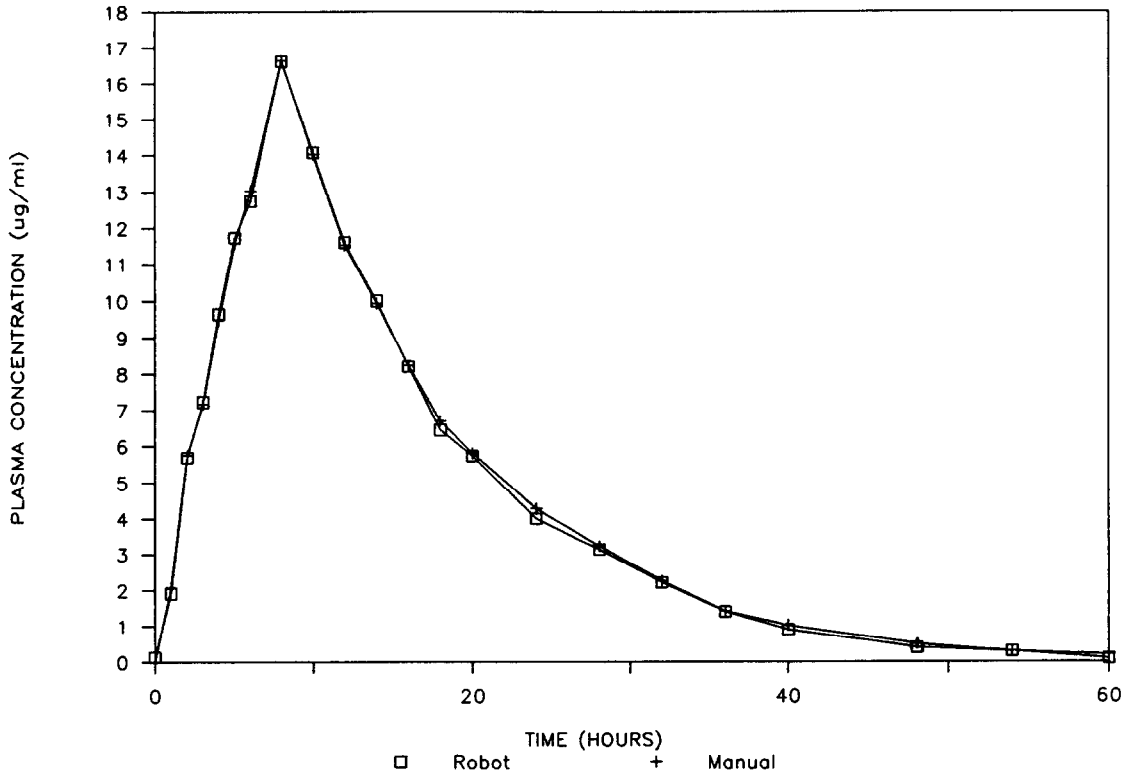

Figure 4
Theophylline plasma concentration versus time curves as obtained after administration of a 800 mg dose (i.v.) as aminophylline-infusion. The concentrations have been measured with both the manual and the automated method.

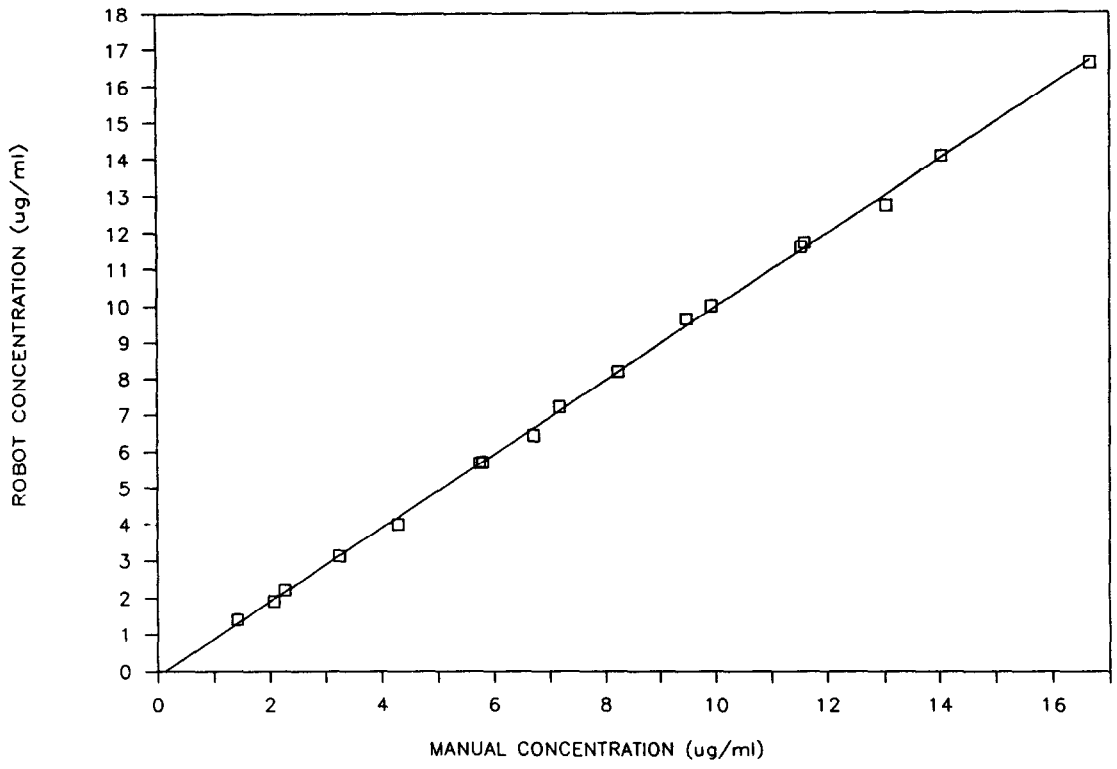

Figure 5
Regression curve of theophylline concentrations measured with the automated method versus concentrations measured with the manual method.

procedure. Figure 4 shows the results obtained with both methods in one plot; the manual and automated method gave identical results. Linear regression analysis was performed on the 17 points in the curves. The resulting curve (Fig. 5) has the following equation:

$$\text{conc. robot} = 1.010\ \text{conc. manual} - 0.122 \quad (r = 0.9996).$$

The relation is linear. Small differences between both methods may be due to a difference in calibration solutions. The 95% confidence interval for the slope, β, is:

$$0.994 < \beta < 1.026.$$

The slope does not significantly deviate from unity, so there is no proportional error. The 95% confidence interval for the intercept, α, is:

$$-0.262 < \alpha < 0.019.$$

This interval includes zero, which indicates that there is no systematic error. It can be concluded that there is a good correlation between the automated and the manual method.

The sample throughput of the robotic procedure is 186 samples per 24 h, about twice the manual procedure (two laboratory technicians, one HPLC).

The automated method for the analysis is reliable. Over a period of 1 week, the robot functioned continuously for at least 6 days. One day was needed for correcting system trouble. So, in 1 week a maximum of $6 \times 186 = 1116$ samples can be analysed by the robot, against $5 \times 93 = 465$ by two laboratory technicians, working 5 days a week.

Conclusions

A robotic procedure for the determination of theophylline in plasma has been developed. The complete sample preparation of plasma samples and the on-line injection of the extracts into the HPLC-system are performed by the robot.

The within day precision and accuracy are very good and comparable to the manual

method. The between day reproducibility of the robotic procedure is, especially at high concentrations, better than the manual procedure. For a less robust technique a robotic method may be much more reproducible than the manual method.

The sample throughput of the robotic method is at least double that of the manual method.

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