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Development of a laboratory robotic system for automated bioanalytical methods — II. A robot computer program for guarding totally automated bioanalytical methods

J. WIELING,*†‡ J. HEMPENIUS,* H.J. JEURING,* J.H.G. JONKMAN,*† P.M.J. COENEGRACHT† and D.A. DOORNBOS†

* *Pharma Bio-Research International B.V., P.O. Box 147, 9400 AC, Assen, The Netherlands*
† *Research Group Chemometrics, Department of Analytical Chemistry and Toxicology, University of Groningen, Ant. Deusinglaan 2, 9713 AW Groningen, The Netherlands*

Abstract: The application of fully automated, unattended sample preparation performed by a laboratory robot for the analysis of drugs in biological samples requires the prevention of system failures which may arise in the on-line coupled chromatographic system or in other components of the robotic system.

A computer program has been developed which can help to detect such problems. The control program for the robotic sample preparation contains a number of safety measures to intercept robotic or human errors. A routine is implemented, guarding for chromatographic malfunctions and errors in dispensing liquids by the robot. After detection of trouble, sample preparation is interrupted.

Keywords: *Robotic sample preparation; robot control program; guarding routine for chromatographic malfunctioning and liquid dispensing trouble; HPLC.*

Introduction

In part I of this paper [1], the validation of a robotic system has been described. The system was used for automated sample preparation of biological samples for HPLC-analyses. The authors concluded that a laboratory robot was very well suited for sample preparation and on-line injection of extracts into a HPLC-system.

A major aim of the application of a laboratory robot for sample preparation is "unattended operation" of the robot, i.e. functioning of the robot without human intervention. This requires a number of safety measures to be built which are used to guard against trouble that may arise in any part of the robotic system and the on-line coupled HPLC-system. This is important especially when samples available are small. If there is malfunctioning in the system which cannot be detected by the robot, sample preparation will continue and samples will be lost.

A robot is not able to interpret chromatograms, therefore artificial "eyes" have to be built in the robot control program.

This paper describes the detection of chromatographic trouble, malfunctioning of the Master Lab Station or human errors.

Experimental

Apparatus

A commercially available Zymate II robot system (Zymark Corporation, Inc., Hopkinton, MA, USA) was used. The most important parts of the robotic system are 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). Reagents were dispensed by the MLS into sample tubes, and the stations were switched on and off by the robot control computer. A complete description of the robotic system and the on-line coupled HPLC-system is presented in Part I of this paper [1].

Chemicals

Theophylline (1,3-dimethylxanthine) and the internal standard (β-hydroxyethyltheophylline) were obtained from Sigma Chemical

‡ Author to whom correspondence should be addressed.

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 LE11 0RG, 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).

Robotic method for the determination of theophylline in plasma

In Part I of this paper [1], validation of the robotic procedure compared with the manual method was discussed. For testing the guarding system to be developed, this robotic theophylline assay was also used. A description of the assay method and details on robotic sample preparation is given in Part I.

Safety measures to prevent robotic and human errors

A number of safety measures were incorporated in the robot control program to realize "unattended operation": a check on the presence of the hand by measuring the "reach force"; a check on the presence of sample tubes by reading the "grip force"; a check on the presence of pipettips by reading the "vertical force" and a check on technical trouble in the centrifuge by detecting "vibration trouble" and "hardware trouble". In all cases, human intervention is necessary to remedy the trouble and to continue robotic sample preparation.

Set-up of a guarding system for chromatographic and Master Lab Station problems

To realize complete "unattended operation", the above mentioned safety measures do not suffice to cover all trouble that may occur with the robot or the on-line coupled HPLC-system.

Trouble that might occur in the HPLC-system are for example:

(1) obstruction of the injection system, leading to no injection;
(2) leakage in the system, causing loss of mobile phase containing the analyte and resulting in detection of inferior peaks;
(3) deterioration of the analytical column;
(4) deterioration of the detector lamp, causing incorrect detection.

Being the supplier of reagents for the sample preparation, the MLS is a component in the system that may also cause trouble during analysis. Possible failures of the MLS are:

(1) the flask containing extraction liquid is empty or the supply tubings are obstructed. This can have far-reaching consequences; no extraction liquid is added to sample tubes and the aqueous layer in the tube, containing all biological matrix components, is injected directly into the analytical column. This may result in destruction of the column;
(2) the flask containing the internal standard solution is empty or the supply tubings are obstructed. In this case no internal standard solution is added and no quantitation of the analyte can be performed.

In our robot computer control program the guarding of the HPLC-system and the MLS is based on observing the internal standard peak in each chromatogram. Usually, in assay methods for drugs in body fluids an internal standard is used and every chromatogram contains an internal standard peak, which has approximately the same height and the same retention time in all runs. In every run, therefore, the chromatographic process and functioning of the MLS can be observed.

Data acquisition by the robot

The Zymark Z310 Analytical Instrument Interface is the robot component able to acquire data. The Z310 contains an AD-converter to read analogue signals from the HPLC-detector. The converted signals can be stored and are used by the guarding program. Figure 1 shows the data acquisition of the Z310.

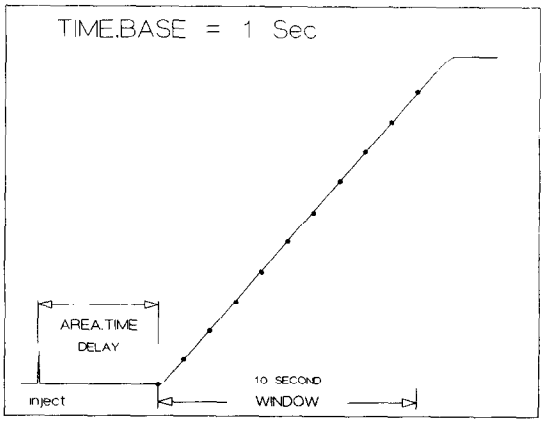

Figure 1
Data-acquisition of the Zymate-robot Analytical Instrument Interface.

During each chromatographic analysis the Z310 can store eight "window" values. These are obtained during a freely adjustable period of time, called a WINDOW, so each chromatogram may contain up to eight windows. A window can start at any desired point of time. The time between injection and the first measurement in a window is called the AREA. TIME.DELAY. All measurements in a window are acquired at a constant time base (TIME.BASE) and up to 100 measurements may be performed within a window. The analogue to digital conversions are summed and the result is registered as a number of counts. The total number of counts that can be collected within one window is 32,768 and this puts a limit to the sampling of the signal.

The algorithm of the Z310 cannot detect peak characteristics such as top or start. It cannot measure peak areas, but collects digital values at specified points of time; these are summed and the result is termed AREA.

Algorithm of the guarding system

The developed program for guarding the HPLC-system and the MLS is based upon the algorithm used by Halloran and Franze [2] and is written in the EasyLab programming language. The program is able to detect trends in chromatographic signals, like baseline drift and loss of sensitivity. The algorithm of Halloran and Franze and that used here differ: The data-acquisition windows of Halloran and Franze are set up automatically by the injection of two standards before analysis. The approximate peak retention time is found after the first injection. The second injection is used to fine tune the window set-up.

The system reported here is used to guard sample preparation and chromatographic analysis of drugs in biological samples. Biological samples often generate many peaks and therefore automatic set-up of the data-acquisition windows is not possible.

From an analytical point of view and for quality assessment, all peaks in the chromatogram are important and data are acquired by an integrator. For the guarding procedure only the internal standard peak is observed by the Analytical Instrument Interface. This gives the programmer the opportunity to use all eight windows for this peak and the more windows used for a single peak, the more accurate a peak measurement becomes. There is no delay time between subsequent windows, and they have equal length and TIME.BASE. After each analysis the baseline drift of the chromatographic system and the retention time and the peak response of the internal standard peak are checked by the robot controller, by means of the Z310.

On starting up, the program asks for the approximate retention time and peak width of the internal standard peak. The algorithm uses the peak width to determine the length of a window which is done in such a way that the peak contains four windows (WINDOW no. 3 up to and including WINDOW no. 6). The first two and the last two windows are used to calculate net peak responses and to detect baseline drift and shift of retention time. They are also used as a buffer for peak shifts. AREA.TIME.DELAY's are determined such that WINDOW no. 5 is the window with highest response, i.e. the top of the peak. Before every new injection the algorithm searches for the window with highest response and if this differs from WINDOW no. 5, then all windows are adjusted in such a way that in the subsequent run WINDOW no. 5 is again the window with highest response.

The Z310 has many restrictions. The total number of counts per window is the only output. Separate measurements within a window cannot be obtained and it is therefore impossible to determine accurate retention times. The retention time is roughly determined by calculating the middle of the window with highest response. Consequently, retention times determined by the robot change in steps of n times the length of a WINDOW (n*WINDOW (n = 1,2,3, . . .)).

The total peak response is calculated as shown in Fig. 2.

Before and after the top of the peak, the windows with lowest responses are determined ("background responses") and their mean is calculated. Then, from any window between these two background responses, the mean background response is subtracted from the AREA's and these corrected values are summed, the result giving the total peak response. In Fig. 2, for instance:

$$\text{TOTAL AREA} = \sum_{i=2}^{7} \text{AREA (i)} - 6 \times \frac{\text{AREA(1)}+\text{AREA(8)}}{2} . \quad (1)$$

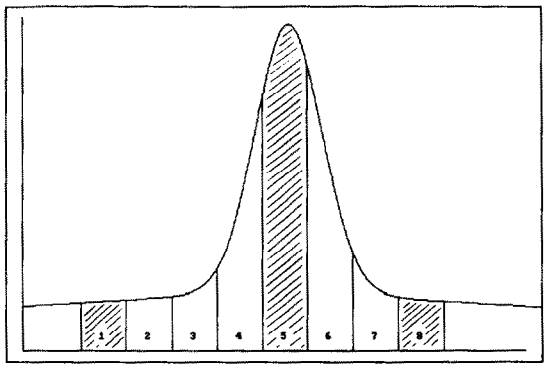

Figure 2
Calculation of total peak response of the internal standard peak. Window no. 1 and window no. 8 are the background response windows. Window no. 5 is the window with highest response. Calculation of the total peak response is by equation (1).

Decision making for qualifying chromatograms

Baseline stability check. Checking the baseline of the HPLC-apparatus before another sample is injected into the HPLC is of vital importance for the good performance of a totally automated sample preparation system. The robot has to terminate sample preparation and injection of pre-treated samples into the HPLC-system when the baseline drift is unacceptably high.

In our program the baseline stability check is built in two stages: (1) before the first sample is injected, the baseline is observed until it is sufficiently stable; (2) preliminary to injection of every pre-treated sample the baseline is observed and its drift is calculated.

WINDOW no. 8 is the baseline reference. After each run its previous value is stored in LAST.BASELINE and the new response of WINDOW no. 8 is stored in BASELINE. Decision making by the robot controller is as follows:

(1) if the difference between LAST.BASELINE and BASELINE is less than 10% *then* the baseline is accepted and the robot can inject the next sample;
(2) if the difference between LAST.BASELINE and BASELINE is more than 20% *then* the baseline is not accepted. The drift is too high and the robot will terminate sample preparation and injections;
(3) if the difference is between 10–20% *then* the robot will warn by printing a message. The robot continues sample preparation and will make another injection. With this new injection LAST.BASELINE is not replaced by BASELINE, but a new BASELINE is obtained from the new chromatogram. This new BASELINE is compared with LAST.BASELINE (=BASELINE of two runs before). If the deviation between the two values is more than 15%, the robot will terminate sample preparation and injection. If the deviation is less than 15% it will continue.

If the robot has decided to terminate sample pre-treatment and injection, a message will be printed explaining the reason for terminating. It will also give a few suggestions for remedying the possible trouble.

Retention time check. As stated earlier, retention time is determined by searching for the window with the highest response (in most cases WINDOW no. 5). Retention time shift is detected when the window with the highest response is not WINDOW no. 5. In the next chromatogram, the windows will be adjusted such that WINDOW no. 5 is the window with the highest response. Decision making for retention time check is as follows:

(1) if the retention time did not shift *or if* the retention time shifted one window *then* the chromatogram is qualified as being acceptable. No shift indicates a stable chromatographic system. One window shift is acceptable also, since in this case the real retention time shift is less than 25% of the total peak width.
(2) if the retention time shift is more than one window *then* the chromatogram is considered unacceptable and the system will wait for a human decision, whether to continue or terminate sample preparation. A message is printed and a few suggestions are given for remedying the possible trouble.

Peak response check. In each chromatogram the peak response of the internal standard is calculated. The program uses the first six chromatograms to calculate the mean and the standard deviation of the internal standard peak. These values are used in the continuing part of the guarding program as parameters for checking the peak response. Decision making by the robot is based on the Shewhart chart in Fig. 3. Peak responses exceeding the upper and lower control limits are graded as being not accepted and the robot will terminate sample preparation and injection. A message is

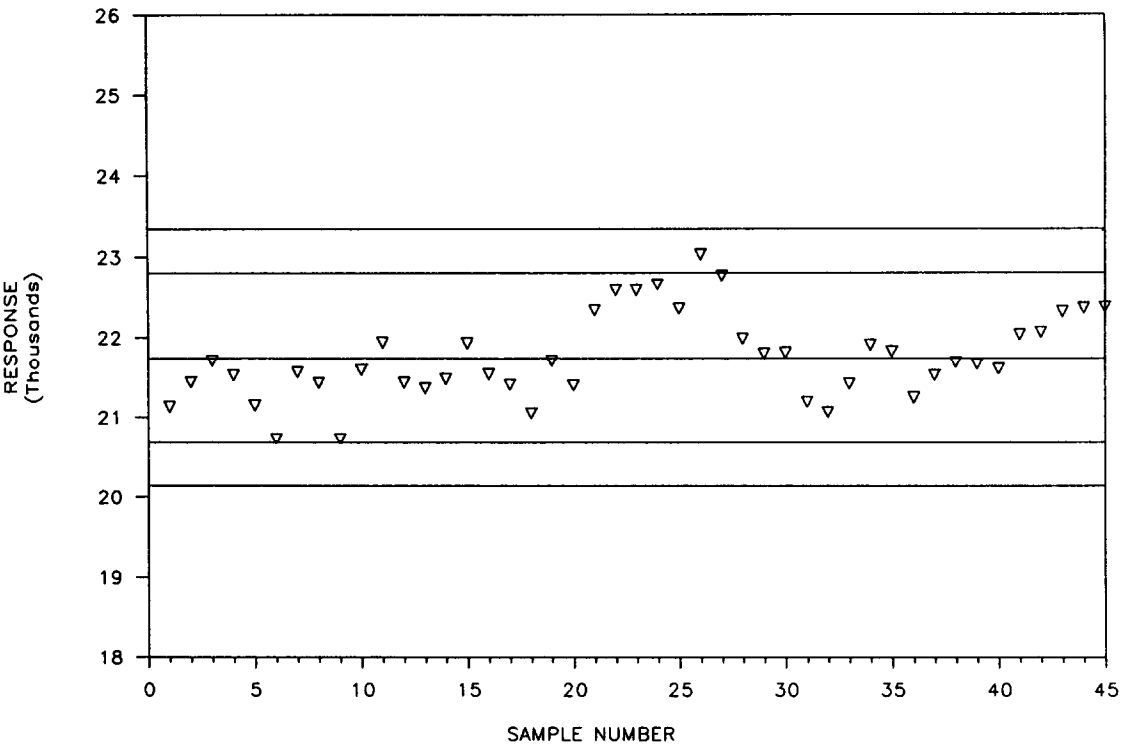

Figure 3
Shewhart chart of calculated peak responses of 45 consecutive theophylline analyses, with β-hydroxyethyltheophylline as the internal standard.

printed in which possible errors in the HPLC-system or the MLS are given. Human decision can give the robot the instruction to continue (after remedying trouble) or to shut down the whole system by simply pressing a key.

Results and Discussion

After implementation of the developed guarding module in the operating software of the robot computer (controller), the algorithm was validated by the robotic pre-treatment of a large number of samples.

Figure 3 gives a Shewhart chart of 45 consecutive analyses and shows that calculation of peak responses in accordance with equation (1) is valid. The upper and lower warning limits include 44 of the 45 samples and none of the measurements exceeds the upper or lower control limit. Supposing normal distribution, this indicates that 99.7% of the calculated peak responses are within the upper and lower control limits.

To investigate the linearity of the calculation of the peak responses, a calibration curve of theophylline in plasma was made. Here, theophylline was used as the internal standard, so the guarding program calculates the peak response of the theophylline peak in the chromatogram. A curve of calculated peak responses against concentration using the Z310 (correlation coefficient $R = 0.9999$) showed comparable correlation with a calibration curve made with a Spectra Physics SP 4270 integrator ($R = 0.9999$). Linear regression analysis was performed on the six points in both curves. The resulting curve had a correlation coefficient of 0.9998. The small intercept (74 Z310-counts) showed that there was no systematic error.

Conclusions

A robotic procedure has been developed, which takes care of complete bioanalytical HPLC-analysis of drugs in biological matrices, including preparation of plasma samples and on-line injection into the HPLC.

The system operates unattended and monitors correct functioning of the HPLC-system and the Master Lab Station. After detection of trouble in these components, the controller stops the robot arm action and interrupts sample preparation and injection.

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