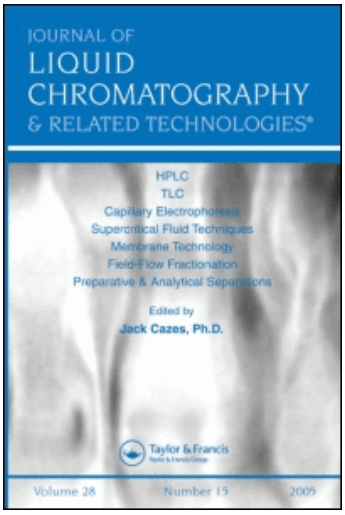

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An Automated Method for the Determination of Enoximone and Its Major Sulfoxide Metabolite in Plasma Using Robotic Technology-High Performance Liquid Chromatography

S. M. Walter[a]; K. Y. Chan[a]; J. E. Coutant[a]
[a] Department of Drug, Metabolism Merrell Dow Research Institute Cincinnati, Ohio

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AN AUTOMATED METHOD FOR THE DETERMINATION OF ENOXIMONE AND ITS MAJOR SULFOXIDE METABOLITE IN PLASMA USING ROBOTIC TECHNOLOGY - HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

S.M. Walter, K.Y. Chan and J.E. Coutant
Department of Drug Metabolism
Merrell Dow Research Institute
Cincinnati, Ohio 45215

Summary

An automated analytical procedure for the determination of enoximone, a new cardiotonic agent, and its major oxidative metabolite in plasma using robotic technology is described. A Zymark robot is used to perform all the operations of solid phase extraction including column pretreatment, internal standard addition, sample mixing, sample pipetting, column rinsing, drying and sample elution. The processed sample is injected directly into the high performance liquid chromatography system which is equipped with an ultraviolet absorption detector. The assay has good precision and accuracy, equivalent to the manual method it replaces, and yet provides higher throughput of sample.

Introduction

Enoximone, 1-3-dihydro-4-methyl-5[4-(methylthio)benzoyl]-2H-imidazol-2-one(I) (Figure 1), is a novel cardiotonic agent currently undergoing phase

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III clinical trials for the treatment of congestive heart failure, (1-5).

Initially an analytical method utilizing high performance liquid chromatography (HPLC) was used to measure plasma levels of I only (6). Subsequently, another method was developed (7) which could quantify both the parent compound and its sulfoxide metabolite (II) (Figure 1) with one HPLC injection.

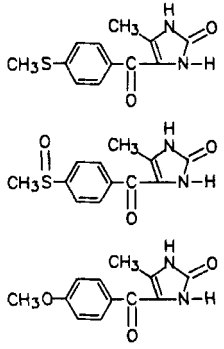


Figure 1. Chemical structures of enoximone (I), sulfoxide metabolite (II), and the internal standard (III).

The large number of samples and time consuming nature of the liquid-liquid extraction procedure used to prepare the samples placed a considerable strain on the analytical capabilities of our department. This paper describes an automated procedure for the quantitation of enoximone and its sulfoxide metabolite in human plasma using robotic technology and solid phase extraction (SPE). This new method provided an expected savings in manpower and an unexpected improvement in precision.

EXPERIMENTAL

Reagents and Chemical

Reagent grade chemicals were used throughout this study. HPLC grade solvents from Burdick and Jackson (Muskegon, MI, U.S.A.) were used to make all solutions. The enoximone (I), authentic sulfoxide metabolite (MDL 19438) (II) and the internal standard material (MDL 82249) (III) (Figure 1) were obtained from Merrell Dow Pharmaceuticals (Cinninnati, OH, U.S.A.). The disposable pipet tips (200-1000 µl), test tubes (16 mm x 125 mm), scintillation vials (20 ml) and Baker 10 SPE C_{18} LD 3 ml columns were obtained from Curtin Matheson Scientific (Houston, TX, U.S.A.). Drug free human plasma was supplied by the Plasma Alliance (Knoxville, TN, U.S.A.).

Instrumentation

The robotic system was obtained from the Zymark Corporation (Hopkinton, MA, U.S.A.). It consisted of the robot arm, system controller, two 5 x 5 scintillation vial racks, two 5 x 10 pipet tip racks, two 5 x 10 3 ml SPE column racks, two 5 x 10 16 mm test tube racks, general purpose had (gripper), dual function hand (gripper and syringe), liquid distribution hand with a nozzle for manipulating SPE columns, master laboratory station, HPLC injection station, balance and two power and event controllers. The evaporation station was constructed from a motorized lab jack and heating block. All of the modules were mounted on a 4 x 7 foot robot workbench. Figure 2 shows the layout of the workbench.

The HPLC system (Millipore, Waters Chromatography Division, Milford, MA, U.S.A.) consisted of a Model 6000A pump and Model 441 UV

detector with a 340 nm filter.

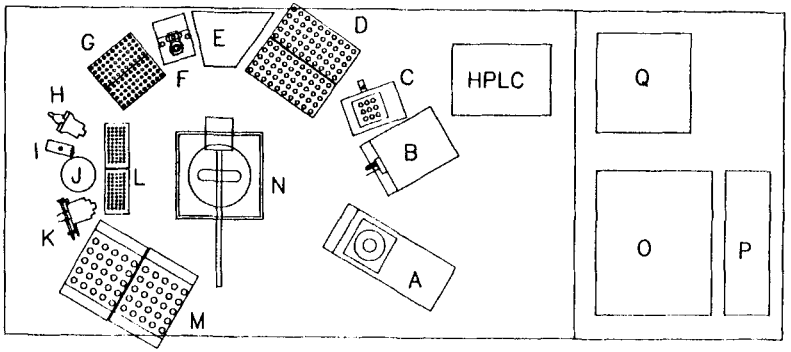


Figure 2. Scale layout of the robot bench where : A = balance, B = HPLC injector, C = evaporation station, D = test tube racks, E = SPE column handling station, F = vortexer, G = SPE column racks, H = gripper hand, I = optical sensor, J = capper, K = Dual function hand, L = pipet tip racks, m = sample vial racks, n = robot arm, O= robot controller, P = robot controller keyboard, Q = printer. The entire bench is 4 x 10 feet.

A 25 cm x 4.6 mm I.D. Zorbax CN (5 µm particle size) HPLC column (Dupont, Wilmington, DE, U.S.A.) was used along with a 2 µm guard filter (Rheodyne, Cotati, CA, U.S.A.) hand packed with pellicular CN packing (Alltech Associates, Deerfield, IL, U.S.A.). The mobile phase was 38/62 methanol/water 0.05M phosphate buffer at an apparent pH of 3.0 with a flow rate of 1.0 ml/min. Beckman CALS laboratory data system (Beckman Instruments Inc., Fullerton, CA,. U.S.A.) was used to acquire, integrate and analyze the chromatographic data.

Plasma Standards

Standard solutions containing equal amounts of I and II were prepared by adding precise aliquots of stock aqueous solutions to drug free human plasma. The standard solutions had concentrations ranging from 3000 ng/ml to 50 ng/ml. These solutions were divided into 3 ml aliquots and frozen until use.

Validation Study

To show the accuracy and precision of the analytical method, 54 randomly coded unknowns containing enoximone(I) and its sulfoxide metabolite (II) were analyzed in duplicate over several days. To test the within day precision, 6 identical sample were assayed on one day. The extraction efficiency was determined by comparing the peak heights found for extracted standards and unextracted standard solutions prepared in the reconstitution solution. The concentration of I and II in the unknowns were chosen to be representative of the concentration ranges previously observed in clinical samples.

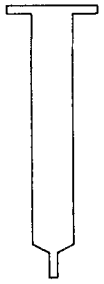
Extraction Procedure

The sample extraction procedure was divided into two parts. Those operations done by the human operator and those operations performed by the robotic system.

The analyst checked all solutions used in the assay and replenished solutions as needed. The HPLC system was checked for proper operation and the racks of disposables (pipet tips, test tubes, SPE columns) filled. The robot was initialized, samples and standards were loaded into racks and the procedure started. No operator interaction was

needed once he analysis was started.

Samples and standards were analyzed by the robot with the following procedure. Each cycle of one extraction started with the liquid distribution hand attached to the robot arm. A clean SPE column was selected from the rack and attached to the nozzle on the liquid distribution hand. Using an optical sensor, it was verified that the SPE column had been attached, the SPE column was moved to the SPE column work station which allows the disconnection and reconnection of the SPE column from the nozzle. The SPE column was preconditioned using two x 2.0 ml of methanol then two x 2.0 ml of water. For each solvent addition, the nozzle was disconnected from the SPE column, solvent added, the nozzle reconnected and the column pressurized with nitrogen for a set time to blow the solvent through. After preconditioning, 1.0 ml of the internal standard solution was added. The robot switched to the dual function hand and obtained the proper sample vial. The sample vial was weighted, mixed and uncapped, the dual function hand was rotated to the syringe operation position and a disposable pipet tip attached. After the pipet tip attachment was verified with an optical sensor, 1 ml of sample plasma was pipetted into the SPE column. The pipet tip was disposed of and the sample vial recapped. The sample vial was reweighed and the mass of sample removed calculated to verify the pipetting operation. The liquid distribution hand was reattached and the sample blown through the SPE column using nitrogen at approximately 10 PSI. The SPE column was then rinsed with two x 2.0 ml of water and dried for 5 minutes with nitrogen. The liquid distribution hand with the SPE column attached was positioned over a clean test tube and the sample eluted with two x 2.0 ml of methanol.



Precondition SPE Column with:
2 x 2.0 ml Methanol
2 x 2.0 ml Water

Add 1.0 ml Int. Std. Solution
and 1.0 ml of Sample Plasma

Pull Solution Through the Column

Rinse the SPE Column with:
2 x 2.0 ml Water

Dry for 5 Minutes

Elute into a Clean Test Tube with:
2 x 1.0 ml Methanol

Figure 3. Schematic of the SPE extraction procedure for enoximone.

The sample was evaporated to dryness with nitrogen using a heating block at 55 C. The sample was then reconstituted with 400 µl of 20/80 methanol/water and 200 µl injected into the HPLC. Figure 3 shows a schematic of the extraction for I and II in plasma.

Calculations and Calibration

The general from of the calibration equation was:

$$Y^n = AX + B \qquad (1)$$

where Y is the peak height ratio (expressed as percent by CALS) found by dividing either I or II by the internal standard,
X is the concentration of compound,
A is the slope of the fitted line and
B is the intercept.

Assuming B = 0, the natural logarithm of both sides was determined and the equation rearranged to:

$$\ln(Y) = \frac{\ln(A)}{n} + \frac{1}{n} * \ln(X)$$

A linear regression on ln(X) *vs* ln(Y) for each standard in the standard curve permitted calculation of n and A from the slope and intercept:

$$\text{slope} = \frac{1}{n}$$

$$\text{intercept} = \frac{\ln(A)}{n}$$

Substitution of these into (1) gives:

$$\text{amount found (ng/ml)} = \frac{\text{peak height ratio}^n}{A}$$

Figure 4 shows typical data for both enoximone and the sulfoxide metabolite linear calibration samples with the calculated function superimposed.

RESULTS AND DISCUSSION

HPLC Conditions

This method was developed as a replacement for the current liquid-liquid extraction method. Several changes were made in the chromatgraphic system to improve performance. To reduce the interferences observed in some samples with the quantitation of II, the UV detection wavelength was changed from 313 to 340 nm. A check of the UV spectra I and II showed that their absorptivity was roughly equivalent at both wavelengths. A new internal standard III was chosen which eluted between I and II.

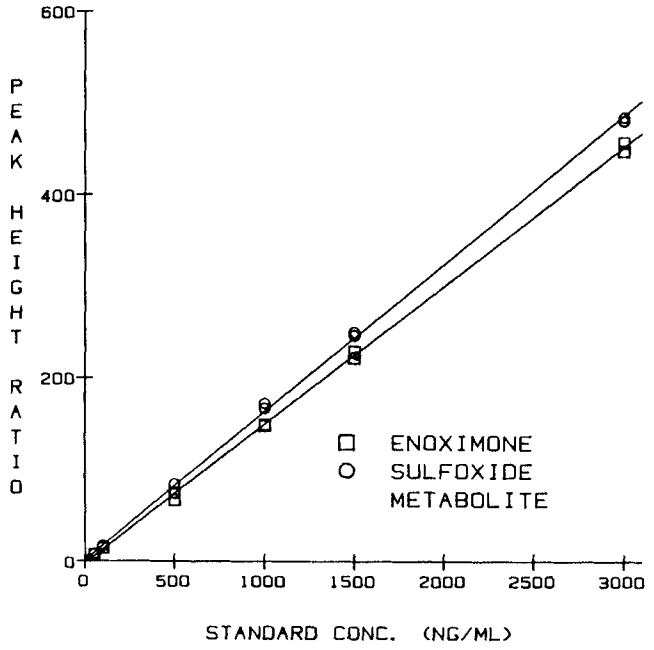


Figure 4. Plot of a typical standard curve and the line fit to the data for enoximone and the sulfoxide metabolite.

This allowed a reduction in the percent organic composition of the mobile solvent to 38% which shifted the retention time of II away from the solvent front and gave retention times of 5.0, 6.7 and 8.7 minutes for II, internal standard and I respectivity. Figures 5 and 6 show some typical chromatograms from extracted plasma samples.

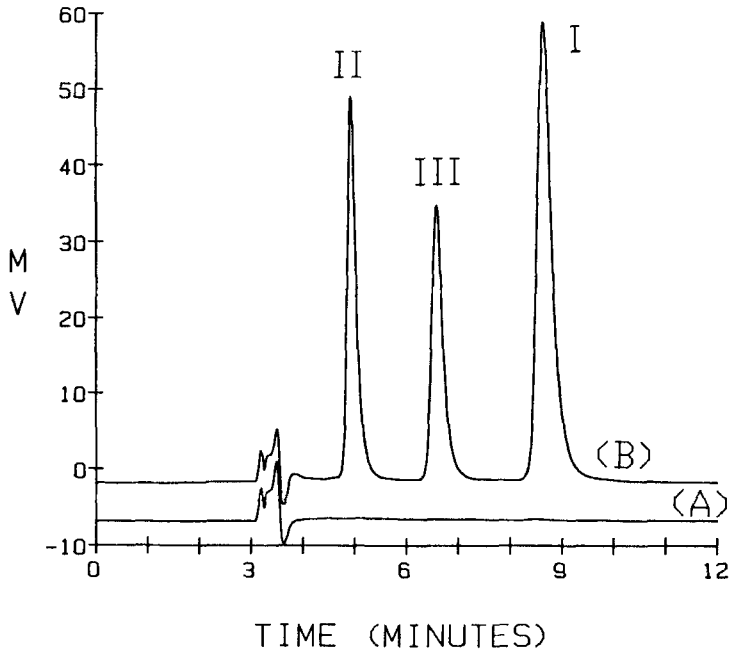


Figure 5. Typical chromatograms for an extracted plasma blank (A) and a 1000 ng/ml (enoximone and II) standard solution (B).

Validation Study

The recovery of enoximone and II from plasma samples was determined by comparing the peak heights of the respective peaks from extracted standards in human plasma to the peak heights of unextracted standards. The extraction efficiency was found to be 100% within experimental error for both I and II. These results are presented in Table I.

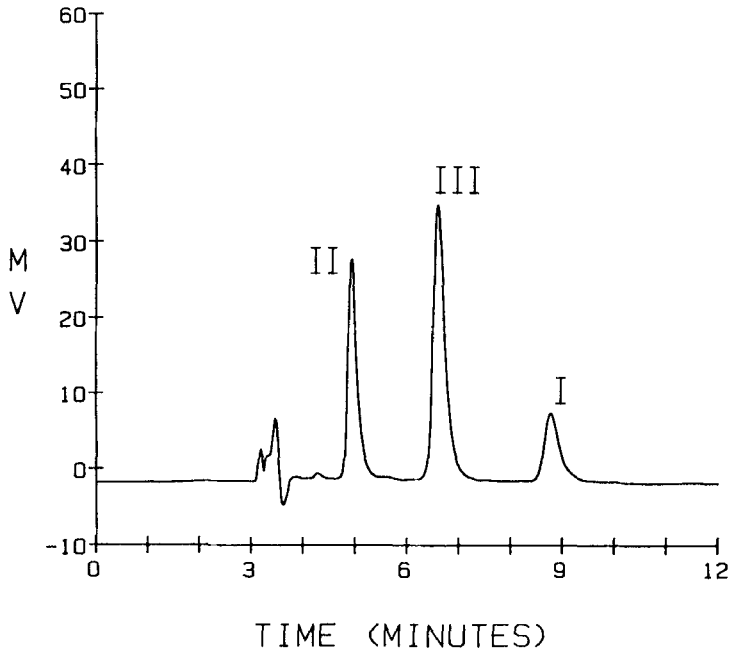


Figure 6. Typical chromatogram for an actual plasma sample. This sample was taken on the second day of a clincal study, 30 minutes after an IV dose (0.5 mg/kg) of enoximone.

The accuracy and precision of the method was determined by assaying 48 randomly coded unknowns on four different days. Although the standard curve includes concentrations from 50 ng/ml to 3000 ng/ml, unknowns were included which had concentrations outside of this range to evaluate the limits of the method. An overall recovery of 100.3% with a relative standard deviation of ±3.4% was found for enoximone. For II, the recovery was 98.3% with a relative standard deviation of ±2.8%. These results are presented in Tables II and III.

TABLE I

EXTRACTION EFFICIENCY RESULTS

	ENOXIMONE	II
Unextracted		
Avg. Peak Height	15.59	14.96
Extracted		
Avg. Peak Height	14.70	13.98
Extraction Efficiency	101.9%1	101.0%

Extraction Efficiency = Ext. Peak Height / Unext. Peak Height / .925*

*The solutions used in this study assumed a sample volume of 1.0 ml. In fact, the robot set the sample volume to .925 ml so a correction factor was needed.

The within day precision of the method was determine by assaying a plasma sample containing 1000 ng/ml of both I and II six times on one day. These results gave a recovery of 98.7% with a relative standard deviation of $\pm$1.4% for enoximone. For II, the recovery was 101.3% with relative standard deviation of $\pm$1.3%. These results are presented in Table IV.

When using the robotic method, plasma samples sit at room temperature for as long as 20 hours before analysis. The stability of enoximone and II in plasma at room temperature was checked by preparing a pool sample and dividing it into several aliquots.

TABLE II

ACCURACY AND PRECISION FOR II

NG/ML ADDED	n	FOUND MEAN	STD.DEV.	REL STD.DEV. %	RECOVERY %
25.0	6	25.1	1.1	4.6	100.6
100.0	6	98.1	2.0	2.1	98.1
250.0	6	246.6	7.6	3.1	98.6
500.0	6	493.1	6.5	1.3	98.6
700.0	6	688.1	14.5	2.1	98.3
1600.0	6	1540.8	56.3	3.7	96.3
2500.0	6	2453.0	62.5	2.5	98.1
4000.0	6	3908.0	41.2	1.1	97.7

Some aliquots were frozen and others were left at room temperature for various time periods up to three days. Relative recoveries were 100% within the error of the method for this time period. Results are given in Table V. The stability of enoximone and II in frozen plasma has been demonstrated in previous work. All of the validation samples were assayed within 24 hours.

Application of the Method

This procedure has been applied successfully to the analysis of samples from single and multiple

TABLE III

ACCURACY AND PRESICION FOR ENOXIMONE

NG/ML ADDED	n	FOUND MEAN	STD.DEV.	REL. STD.DEV. %	RECOVERY %
25.0	6	26.2	1.3	5.1	104.7
100.0	6	99.8	1.7	1.8	99.8
250.0	6	249.6	13.0	5.2	99.8
500.0	6	497.8	11.3	2.3	99.6
700.0	6	691.5	15.6	2.3	98.8
1600.0	6	1582.2	52.6	3.3	98.9
2500.0	6	2507.7	45.5	1.8	100.3
4000.0	6	4033.7	50.4	1.2	100.8

Note: All blank values were 0.0 (n = 6)

TABLE IV

WITHIN DAY PRECISION OF II AND ENOXIMONE

	NG/ML FOUND II	NG/ML FOUND ENXOIMONE
n =	6	6
Average =	1013.3	986.6
Accuracy =	101.3%	98.7%
CV =	1.3%	1.4%

TABLE V

PLASMA STABILILTLY AT ROOM TEMPERATURE RESULTS

SAMPLE AGE (DAYS AT RT)	FOUND*	
	II	ENOXIMONE
3	100.7%	96.9%
1	100.6%	99.9%
0	100.1%	100.1%
0	99.9%	99.8%

*The % found was calculated by taking the average peak height ratio for the zero day old samples and comparing this to the peak height ratios found for the 1 and 3 days samples run at room temperature.

dose, oral and intravenous studies in normal subjects as well as patients with congestive heart failure. Typical results are shown in Figure 7 for a single, intravenous dose of enoximone.

Robotics System

To maximize the throughput of the robotic system, the analysis procedure was broken into 3 overlapping procedures; extraction, evaporation to dryness and HPLC analysis. The extraction procedure required approximately 16 minutes/sample, the HPLC system 15 minutes/injection and the evaporation time for extracted samples was 40 minutes. The evaporation cycle was programmed to take 4 samples at a time and was executed once every four extractions. An HPLC injection was made at a set time during each extraction.

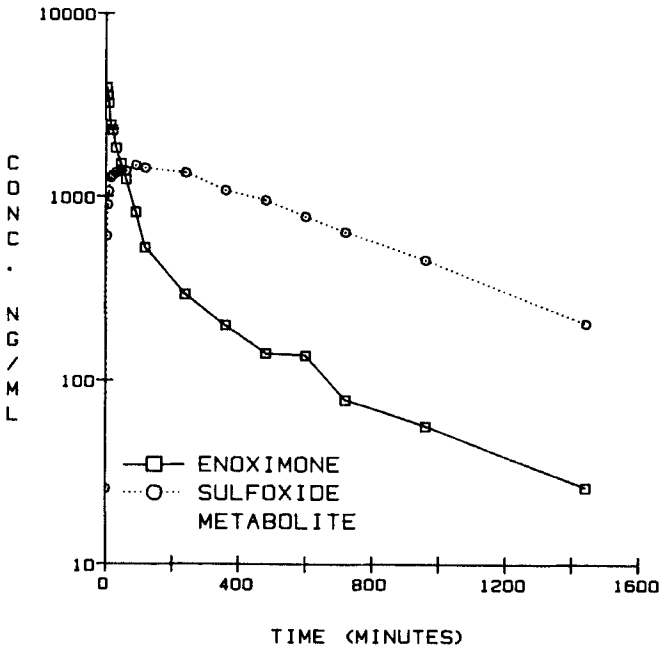


Figure 7. Typical data analysed with the described method. This shows the plasma levels of a single patient as a function of time of enoximone (I) and the sulfoxide metabolite (II) following a single IV dose (1.5 mg/kg) of enoximone.

This gave a sample throughput of approximately 4 samples/hour with a latency of 2 hours from the sample extraction to the sample injection. A typical run which included seven standard solutions which were assayed at the beginning and end of the run and 40 sample solutions which were assayed once, required approximately 20 hours of robot time to run.

The HPLC injection station consisted of an electrically actuated Rheodyne valve with a injection syringe port. It was designed for use with the

syringe hand. The port relied on a friction fit between the syringe needle and the the injection port ferrule to prevent leakage of the sample during loop loading. The speed of the syringe discharge could not be controlled which led to leaking during loop filling due to the back pressure. The station was designed to be used with a robot controlled syringe hand or for direct filling from the elution of the minicolumn. This system was not suitable for small volume (<200 µl) sensitivity. An alternative system which used a syringe on the master laboratory station and a sipper tube on the injector proved to be much better. A bevel on the end of the sipper tube along with it's flexibility ensures that almost 100% of the reconstituted sample could be aspirated. By compensating for the volume of the sipper tube, 100% of the sample could be loaded into the loop. Software control of the aspiration syringe also allows partial loop injections. The 2-way valve on the syringe allows the back flushing of the injection loop and sipper byte with solvent to eliminate carry over between samples.

The evaporation station was designed using a motorized laboratory jack which could be slowly raised over time to speed the evaporation of large volumes of solvent from filled test tubes. the instability of the laboratory jack due to poor mechanical construction and inopportune operator errors lead to concern about the reliability of this system for sample evaporation. A second evaporation station was installed after the validation study which used a pneumatically controlled manifold for the nitrogen probe control. This was less flexible in that only one probe height was possible, but much more reliable.

The physical interface between the SPE column cartridge and the nozzle used for SPE column transport, liquid delivery and nitrogen pressurization was a continuing source of problem. A significant amount of force was required a reliably attach the nozzle to a SPE column. At times, several columns in a row would fail to attach. The seal between the nozzle and the cartridge was poor at times which caused leaking when the system was pressurized to push fluids through the SPE column. This caused serious problems which adversely affected the extraction procedure and chromatography. A separate robotic work station has been designed to handle SPE columns in a much more reliable fashion and has just recently been implemented on this robot system. This SPE column is placed in the station after which the nozzle is pneumatically driven to seal the cartridge. No problems with pressurization or lost cartridges have been observed with this station. It also considerably reduces the amount of hand movement required by the robot arm. Included in the station is a mechanism for placing a waste funnel or a testtube directly under the SPE column. This allows the entire extraction procedure to be done without need of the robot arm.

Several components of the HPLC system were monitored and controlled by the robotic controller. A solvent select valve was used to rinse the HPLC column at the end of each urn. The HPLC system power was also robot controlled. This allowed Friday night runs with no operator checking before Monday. Before each injection the column back pressure was checked. if the pressure was out of limits (high or low) the HPLC system was turned off and no injections were made although sample processing continued. Separate routines allowed the injection of processed samples after the HPLC problems have been solved. The

Beckman CALS system was also checked for readiness before every injection.

Manual vs. Automated Method

A validation of the manual execution of this method was also done to compare with the robotic results and to allow sites without robots to run the method. The results for 54 randomly coded unknowns gave an average recovery 98.0% with a relative standard deviation of ± 2.5% for enoximone. For II, the average recovery was 97.3% with a relative standard deviation of ± 1.8%. These results are comparable with those obtained by the robotic system.

Each analyst that performs a manual sample preparation procedure must be tested to show that results comparable to those obtained by the original analyst are possible. This testing can take up to a week to do. One of the advantages of using an automated method is that all of the technique sensitive aspects of the assay, e.g. pipetting, rinsing and reconstitution are performed by the robot. If the operator makes a mistake, there will be a catastrophic failure of the system and no results will be generated. Therefore, the statistics generated by the original analysts validation will be valid for all operators and different operators may run the system without individual validation.

Another advantage of using robotics and SPE column technology for an automated system is the ease of method develoment. If the chemists developing the analytical methods for new compounds are aware of the limitations of the automated techniques, they can develop manual methods using the SPE columns for initial studies. Then, if the compound enters large scale tests, the manual method can be adapted to the

robotic system very rapidly. All of the method development work, except the final precision and accuracy tests, can be done manually leaving the robot free to continue routine analyses.

In drug metabolism, the sample volumes are limited and precious. Verification techniques are important in reducing the chance of wasted sample and bad results. It is the ability of an automated system to produce higher quality data by eliminating mistakes due to sample handling that is an important advantage. Every time that an object is picked up by the robot, the operation is checked. If an error is detected, the robot can execute a variety of responses. For example, if the attempt to attach a pipet tip to a syringe fails, the robot can try again. If the second attempt fails, the robot will advance to the next position in the pipet tip rack and try again. This sequence will continue until the operation is successful or the end of the rack is reached. An end of rack condition will cause the robot to abort the current operation, complete any tasks that it can and shut down the system in an orderly fashion. Error detection and correction is limited only by the imagination of the robot programmer. Optical sensors can be used to verify that pipet tips and SPE columns are attached to the robot hand. Although this particular robot is not equipped with tactile feedback, a method has been described (8) for checking the finger motor current making it possible to determine whether the robot has an object in it's grasp. A second generation of robots are available which have tactile feedback built in. The verification of the sample pipetting operation is also vital to the operation of the robot. Due to limited sample sizes, the volume of sample may be less than that specified in the procedure. An accurate measure of the volume

pipetted into the SPE column allows computational corrections for limited samples. Optical means of determining the transferred volume are theoretically possible but have practical problems. This system weighs the sample vials before and after the pipetting operation and calculates the volume of sample removed. Since the density of plasma is relatively constant, this relates directly to the volume removed.

The use of such robotic systems with care, planning and attention to error checking can result in systems that not only have good accuracy and precision but also have a high degree of reliability in the identity of the sample. It is the practice of this drug metabolism group to assay all samples determined by manual procedures in duplicate if possible to minimize errors in sample handling that sometimes occur even with a skilled and careful analyst. Samples analyzed on the robotics system were originally run in duplicate until statistical analysis of duplicate analysis showed that they constantly agreed within the limits of the validation study. For many studies the decision was made to analyze samples only a single time using the robotics system and thus dramatically increasing the throughput of the system, a factor of 2.5 over that of the liquid-liquid manual method it replaced.

Future Work

Presently, the interaction between the robotic system and the laboratory data system is very limited. The robot system checks to see if the data system is ready before each injection and starts the integrator. The robot also passes a 2 digit sample identifier (sample vial number) with each injection. A more sophisticated interface is in the design phase

which will allow the robot to pass other sample information to the data system such as sample weight or a bar code read from each sample vial. In addition, information about samples previously injected will be passed back to the robot from the data system. This will be especially useful for methods with limited concentration ranges which may routinely require sample dilution or concentration on some samples before analysis. Returned parameters such as resolution factor or theoretical plate counts could also be useful in methods that place high demands on the chromatographic performance of the system.

Conclusion

A solid phase extraction procedure which is executed using robotic technology has been implemented for the quantitation of enximone and it's sulfoxide metabolite, II, in human plasma. During the first year of operation, 1500 samples have been analyzed using this system limited only by the supply of samples. Due to the success of this technology, we have been able to increase the reliability of our results and keep pace with the clinical program of enoximone with a decreased manpower committment in spite of an increased demand for sample analysis. By the end of 1986, three robots are expected to be operation in these laboratories analyzing a variety of different compounds but using essentially the same concepts presented here.

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