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AUTOMATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SAMPLE CLEAN-UP FOR MASS FRAGMENTOGRAPHIC ASSAYS

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SUMMARY

A previously described high-performance liquid chromatographic (HPLC) sample clean-up procedure has been automated by attaching a (DuPont) auto-sampler and a time-controlled fraction collector to the HPLC equipment. To obtain the required reliability for unattended operation, the sample intake was controlled by volume rather than by time, and the system was protected against sample loss due to non- or improper operation of the injection valve. The capacity of the system depends on the HPLC run time per sample but varies from 45 to 135 samples per 24 h. The recovery and reproducibility are comparable to the manually operated system, while carry-over to subsequent samples is prevented by intermittent injection of the HPLC solvent system as flush fluid.

INTRODUCTION

In a previous paper [1] the use of HPLC for sample clean-up in mass fragmentographic assays was described. It was demonstrated that the recovery of the compound to be determined and the extent of purification was improved when compared with a clean-up by a back-extraction procedure. Additional advantages of the HPLC procedure mentioned were the increased total analysing capacity of up to an average of e.g. 60 samples a day and the possibilities for automation. The present paper describes the set-up and performance of an automated HPLC clean-up equipment for unattended 24-h operation.

MATERIALS AND METHODS

Equipment

The major components of the automated HPLC system are:

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(i) A DuPont 834 Automatic Sampling System (auto-sampler; DuPont, Wilmington, Del., U.S.A.) equipped with the low-volume sample option and a pneumatically operated 6-port $48 \cdot 10^6$ Pa injection valve (Rheodyne, Berkeley, Calif., U.S.A.). The volume of the injection valve loop is 500 μl.

(ii) A Waters Assoc. (Milford, Mass., U.S.A.) Model ALC 202 high-performance liquid chromatograph equipped with a 30 cm × 4 mm I.D. stainless-steel column filled with μPorasil (10 μm; Waters Assoc.). The elution solvent was composed of *n*-hexane—isopropanol (80:20, v/v) to which 4% of ethanol and 0.1% of concentrated ammonia were added. The standard UV detector was operated at 280 nm.

(iii) A Gilson Microcol TDC 80 fraction collector (Gilson, Villiers-le-Bel, France) for 80 tubes, with built-in drop counter.

(iv) A Kipp BD 9 (Kipp, Delft, The Netherlands) two-pen potentiometer recorder with remote control of chart drive and electrical pen lift.

System design

The design of the automated system is schematically illustrated in Fig. 1. The injection valve is connected by 0.25 mm I.D. stainless-steel capillaries to the HPLC pump and column. A drop counter (fraction collector accessory) is connected to the drain outlet of the injection valve.

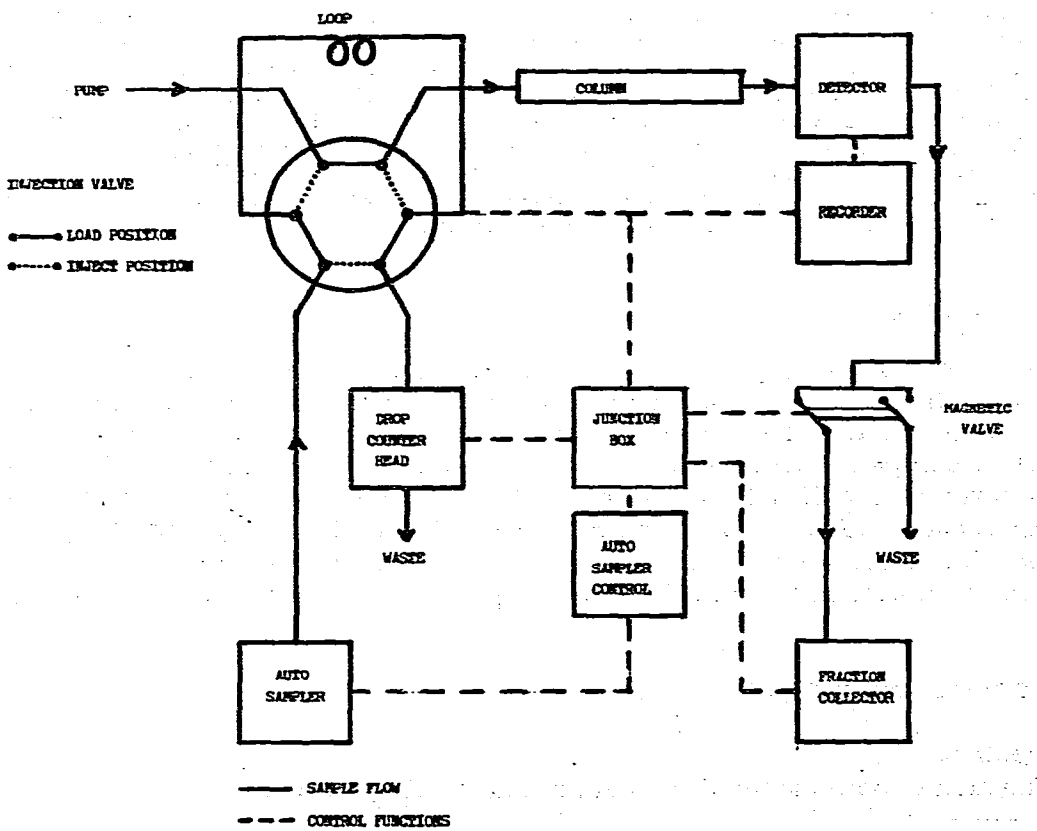

Fig. 1. Design of automated HPLC system for sample clean-up.

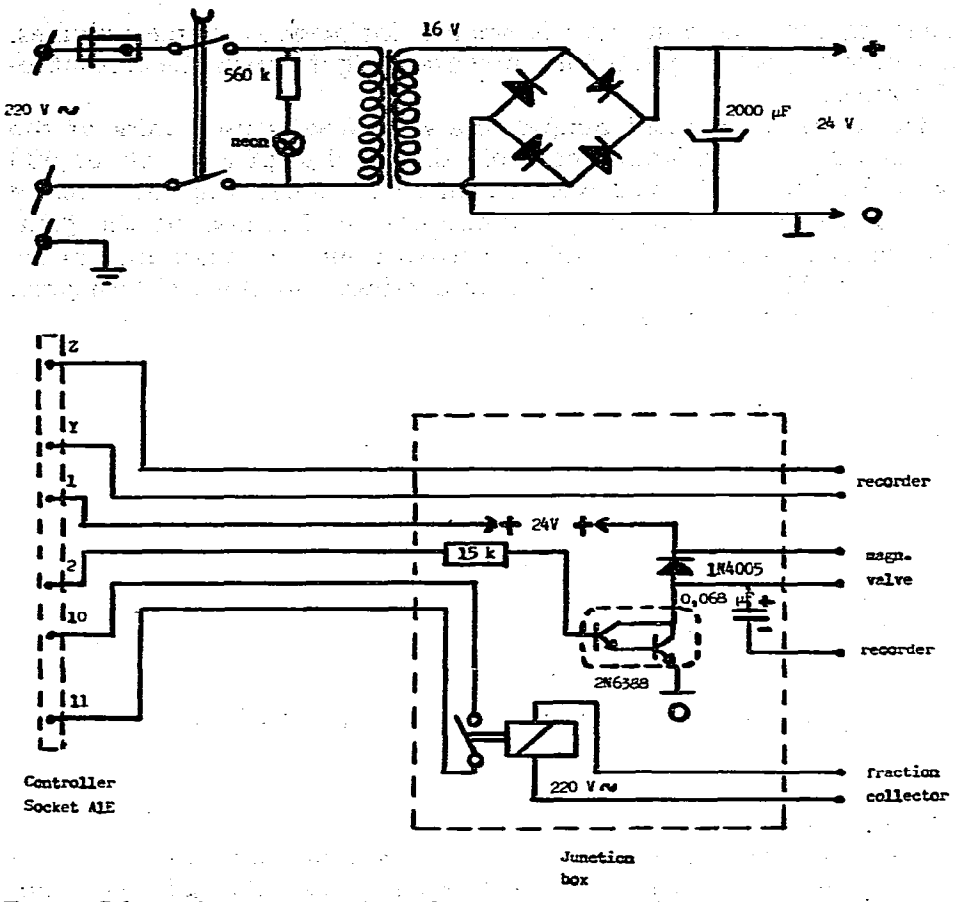

Fig. 2. Schematic representation of junction box and interconnections between the components of the automated system.

The column outlet is fed to a T-piece of which the two other sides are connected by flexible 0.5 mm I.D. tubing to the fraction collector and waste flask. A magnetically operated valve (under control of the auto-sampler timer) blocks either of those two routes and effectively directs the eluate flow either to the sample collection vial or to the waste.

The interconnections between the auto-sampler, magnetic valve, fraction collector and recorder are made via a home made junction box of which the electronic circuit is shown in Fig. 2.

The main power supply of the HPLC-pump is switched by a 24-h timer to enable pump switch-off at a predetermined time after having processed the last sample.

In general, HPLC and also gas chromatographic (GC) auto-samplers are used to inject a small volume, e.g. 2 μl from a larger sample volume, e.g. 2 ml. For the present preparative purpose, however, as much as possible of the sample should be injected, while the maximum injected volume is determined by the minimum required chromatographic performance. Obviously, the relative loss of sample as residue in the sample vial, in the injection needle and

in the capillary to the injection valve is smaller for greater sample volumes. Injection of volumes greater than 0.5 ml, however, might impair the chromatographic performance.

To minimize losses by residual sample, the sample vials used were of the shape shown in Fig. 3. By injecting no more than 450 µl of a sample of 500 µl, the narrow end tip of the vial will remain filled, preventing air entering the system and causing a 90% sample consumption. Because of the great variability in the dimensions of the DuPont narrow-tipped sample vials, causing not only irreproducible sample losses, but also failures in the vial transport, narrow tolerance home-made vials were used.

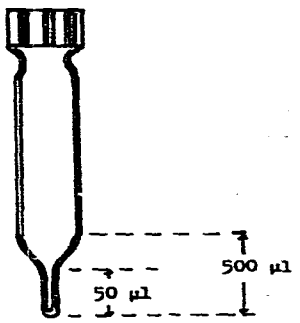

Fig. 3. Screw capped (PTFE rubber laminated septum) sample vial for a DuPont auto-sampler.

Operating procedure

Plasma extracts are evaporated to dryness. The residues are redissolved and transferred into the auto-sampler sample tubes (see Results and discussion for tube specifications) by means of two volumes of 250 µl each of the HPLC elution solvent. When necessary, flush vials (distinguished by their white caps) containing pure elution solvent can be placed intermittently with the black-capped sample vials in the rack.

The drop counter is adjusted to, e.g., 35 drops, corresponding to 0.42—0.45 ml. The auto sampler control timers "start integrate", and "stop integrate" (originally meant to control an auxilliary integrator) are set for the desired start and end time of sample collection. The "total time" controller is set to define the total run time per sample.

After starting the system, the following sequence of operations (illustrated in the time diagram of Fig. 4) is carried out automatically. The first sample vial is brought into position and the tube guiding head of the fraction collector moves to the first tube. The dual injection needle system moves down and pierces through the PTFE rubber laminated septum into the sample vial, down to approximately 1 mm from the bottom of the narrow tip. The vial headspace is pressurized up to $240 \cdot 10^3$ Pa to flush the sample through the injector loop. When the preset number of drops of elution solvent is measured by the drop counter at the injection valve drain outlet, the valve is switched to the inject position, the vial headspace is depressurized and the analysis time counter starts. At the preset "start integrate" time the magnetic

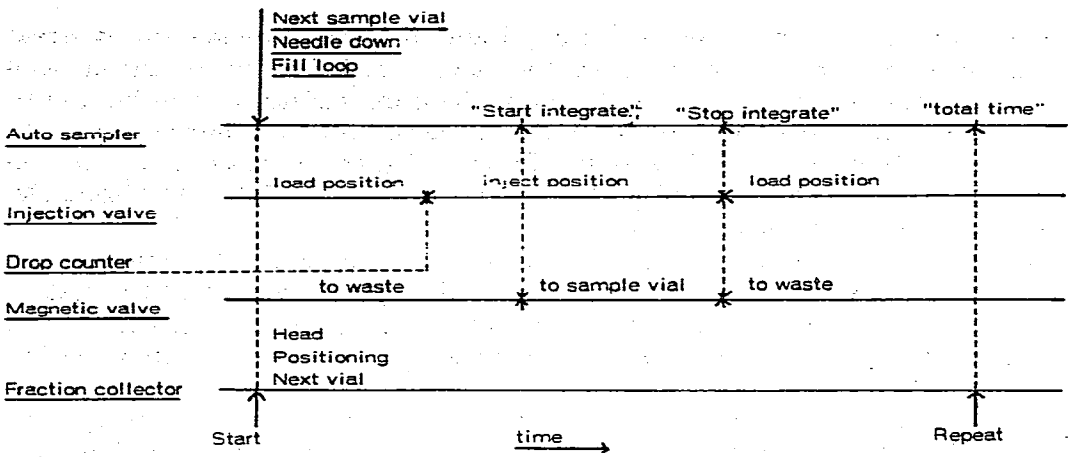

Fig. 4. Time diagram of different system functions for one sequence.

valve opens the column effluent tube to the fraction collector and closes the way to the waste. At "stop integrate" time the magnetic valve is switched again to direct the flow to the waste and blocks the way to the fraction collector tubes. At the same time, the injection valve is reset to the load position.

At the preset "total time" the injection needle system is moved upwards, and a new sequence is started. When flush samples are used, the injection valve is not operated and the subsequent sample is brought forward after the preset volume (equal to the sample inject volume) is flushed through the loop.

RESULTS AND DISCUSSION

Reliability

Auto-samplers are introduced for economic and quality reasons. It is expected that they increase the total analyzing capacity and reduce dull and time-consuming work, while their reproducibility may improve assay quality and may reduce mistakes.

A straight forward connection of the standard DuPont auto-sampler to the HPLC-equipment caused a number of problems resulting either in complete loss of samples or caused failures requiring reprocessing of saved samples. Some of these problems are, however, inherent to the specific use for preparative handling of biological samples. Because the advantages of automation are only achieved when the system can be operated unattended, the required modifications are discussed.

In the normal operating procedure for the DuPont auto-sampler, the injection loop is not filled by controlling the volume of fluid at the drain outlet, but by pressurizing the head space of the sample vials for a preset time. This procedure, however, cannot be used for routine analyses of biological samples. In normal practice, the flow resistance in the line from injection needle to injection valve drain outlet, as well as the head-space pressure will vary from day to day, or even from sample to sample. This variability causes irreproducible volumes of injected sample. The extremes are that, at low resistance and/or

high pressure, the sample vials are emptied and air enters the system whereas at high resistance and/or low pressure the valve is switched to the inject position before the loop was adequately filled. Pressure variations may be due to leaking vial septa or sample pump solenoid valve and to a minor extent to pressure variations in the air supply. Flow resistance variations are caused by deposits in the capillary and injection valve as a result of the use of relatively crude extracts of biological material.

Measuring the injection volume, instead of operating in the time-controlled mode circumvents these problems, although regular cleaning of the system by flushing with appropriate solvents or by injecting alternately sample and rinsing solvent, remains necessary for maintenance of proper system performance.

Owing to normal wear or to accidental failure in the pneumatic system, the injection valve sometimes fails to operate, and the samples will be lost because the sample vial transport and sample intake systems will continue operation. The system can be safeguarded against this fatal failure by mounting a micro-switch under the injection valve, which switches off the auto-sampler when the injection valve does not rotate while the analysing time-counter is running.

Failures in the auto-sampler vial transport and needle injection system will only result in loss of capacity while saving the samples, as long as no injections were made. When the injections were made at incorrect times (which sometimes occurs with our auto sampler, probably due to electronic failures) all samples are lost. Unfortunately, the system cannot be protected against this rarely occurring but fatal failure.

Recovery

The recovery of the automated clean-up procedure in daily routine operation has been determined by processing blank plasma extract spiked with tritiated mianserin [2] (20 nCi, 50 ng per injection) and measuring the amount of radioactivity in the vials of the fraction collector. In this set-up, the recovery was proved to be (67.4 ± 2.8)% (mean ± S.D., n = 17). The maximum possible recovery is determined by the injection and sample collection efficiency. For the manually operated system, it has already been shown [1] that the recovery of injected material is 100%. Any reduction of recovery should therefore come from the injection side. As a minimum of 30—50 μl of the sample solution should remain in the vial tip and because of the total capillary volume from needle to injection valve of 30 μl, the minimum loss of sample equals 80 μl. With a sample and loop volume of 500 μl the maximal recovery therefore equals approx. 85%. Although this would be sufficient for most applications, the recovery could be improved by using a 1 or 1.5 ml sample and loop volume.

Only in case the retention time of the compound to be analysed is shifted during sample processing, the recovery might be reduced because the compound might no longer be completely eluted within the predetermined fraction-collection period.

Retention time variations which are not notified (by shifting retention times of marker compounds) in unattended automated systems, do occur in

daily routine analyses, e.g. by temperature variations. To minimize the risk of collecting the wrong fraction the collecting interval should not be chosen too short or the magnetic valve should be triggered by the appearance of a marker peak.

Carry-over

The carry-over, or contribution to the next sample was determined to be less than 0.1% for the manually operated system. When the automated system is operated without vials containing flush solution, the carry-over is determined by the contents of the injection needle and PTFE capillary to the injection valve. As this volume is 30 μl, the minimum carry-over to the next sample is 6% for a sample of 500 μl. For samples of more or less the same concentration this might be acceptable. In case of a greater concentration variability, however, this carry-over will yield unacceptable inaccuracies and flush solution vials must be used.

Reproducibility

When dealing with internal standards, the reproducibility of the injection is not critical. In case no internal standard is used, the overall assay reproducibility is also determined by the injection reproducibility. Because the reproducibility of the injected volume is ±1 drop, the use of a narrow bore capillary results in small droplets of approx. 15 μl at the drain outlet, corresponding to an injection reproducibility of ± 3.5% at an injected sample volume of 420 μl. This injection reproducibility is in good agreement with the overall reproducibility of 4.2% found in the recovery experiments in a set-up of daily routine processing of tritiated mianserin (vide supra).

Capacity

The capacity, expressed as number of samples which can be automatically processed per unit time, depends upon the retention time of the compound to be measured and the retention times of co-extracted endogenous material which should be eluted prior to the injection of the next sample.

The capacity is illustrated by two different assay methods. One is the relatively simple and fast analysis of the antidepressant mianserin (Bolvidon®) in human plasma [2]. Because of the relatively clean hexane extract used in this method, one complete HPLC run takes no more than 10 min yielding a capacity of approx. 6 samples per hour. When flush samples are used, one auto-sampler rack can contain 45 real samples which can be processed in 8—9 hours.

For the assay of the anti-arrhythmic amino steroid Org 6001* [3], however, an ethyl acetate extraction is used causing co-extraction of interfering impurities with HPLC retention times of up to 0.5 h. In the manually operated procedure the elution of these impurities can be speeded up by increasing for some minutes the flow-rate from 0.7 ml/min to e.g. 4.7 ml/min after the appropriate eluate fraction has been collected. In the automated procedure the total HPLC run time should be 0.5 h per sample, because the flow-rate

*3α-Amino-2β-hydroxy 5α-androstan-17-one-hydrochloride.

cannot be controlled by the auto-sampler. Although in this case the capacity is only 2 samples per hour, the number of samples which can be processed unattended outside normal working hours (8 h) is 30—35, while the total 24-h day capacity equals 45—50 samples, which is about the working day capacity of the GC—mass spectrometric equipment.

CONCLUSIONS

Attachment of an auto-sampler and a time-controlled fraction collector to a high-performance liquid chromatograph results in an automated system for the clean-up of extracts of biological samples, which can process unattended 45—135 samples per 24 h, depending on the required HPLC retention time.

Intermittent processing of flush samples, automatic volume control of sample intake and provisions against sample loss owing to non- or improper operation of the injection valve enable unattended (e.g. overnight) operation with recoveries and reproducibilities comparable to the manually operated system.

ACKNOWLEDGEMENTS

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