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Note

Automation of a complex high-performance liquid chromatography system

Procedures and hardware for a vitamin B_6 model system

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High-performance liquid chromatography (HPLC) provides a powerful tool for the separation and quantitation of biologically important compounds that are similar in structure. The incorporation of microprocessors into HPLC systems provides routine control for such functions as eluent changing or mixing, sample injection, integration control, etc. Because microprocessors have the ability to control many different functions, physical parameters that are not normally altered can now be easily changed to effect greater resolution of compounds. Similarly, changes in detector settings can also be controlled by the same microprocessor to provide maximum sensitivity for each compound as it elutes from the column.

It is the purpose of this note to outline a unique and simple automated system which is microcomputer-controlled[1] and which permits continuous operation. In brief, the microcomputer controls the sample injection, all valves that must be changed during the course of a chromatographic run and a spectrophotofluorometer, thus permitting automatic optimization of excitation and emission wavelength, photomultiplier gain, amplification, and zeroing. It also automatically starts the integrator which computes the concentration levels for each unknown from the knowledge of an internal standard concentration and a previous calibration.

To give a concrete example of the complete automation of a complex system, a recent HPLC system[2] for the determination of all six forms of vitamin B_6 and pyridoxic acid will be used. In order to accomplish complete separation of these seven compounds plus an internal standard (3-hydroxypyridine, abbreviated HOP) in a relatively short period of time, two columns maintained at different temperatures are utilized. During the first portion of each run, four vitamers of B_6*, PMP, PM, PNP, and PN, respectively, are resolved on a column maintained at 55°C. An additional

* Vitamin B_6 compounds have been abbreviated, according to published recommendations (IUPAC–IUB Commission on Biochemical Nomenclature, 1970) as follows: PMP = pyridoxamine phosphate; PM = pyridoxamine; PNP = pyridoxine phosphate; PN = pyridoxine; PLP = pyridoxal phosphate; PL = pyridoxal. PIC has been used as an abbreviation for pyridoxic acid while HOP is used for the internal standard, 3-hydroxypyridine.

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vitamer, PLP, plus HOP are eluted from this column and sent through a second column, maintained at 18°C, for further separation. After these two elute from the second column, the final vitamer, PL, elutes from the first column. Finally, with a buffer change, PIC is made to elute from the first column. Early in these studies it was found that excitation and emission wavelengths of the fluorometric detector must be changed during a chromatographic run to obtain maximum sensitivity for each vitamer or metabolite. These changes were essential in order to quantitate some of the B_6 vitamers in modest volumes of human plasma.

The system required constant operator attention as described. Thus, as extraction procedures were developed for foods[3], biological tissue[4], and plasma[5], the numbers of samples that needed to be analyzed necessitated the incorporation of automatic sample injection and system control so that the chromatograph would run unattended for long periods of time and the operator would be free for other laboratory procedures such as sample preparation.

In addition to a description of the automated system, a brief discussion will be included on increasing the sensitivity, on sampling procedures for very small vitamer level concentrations (1–2 ng/ml) and on decreasing sampling time as these are considerations in some practical applications of B_6 analyses as well as other HPLC analyses.

EXPERIMENTAL*

Complete system

The complete system follows the general guidelines laid down by Beecher[6] in the development of an automated amino acid analyser, but with the addition of microcomputer control. The principal components are outlined in Fig. 1. The pump draws buffer from one of two reservoirs, and passes it through a depulser and multisample inject system (MSIS) into a two-column chromatographic system and finally to a fluorescence detector and then to waste. The detector signal is picked up by a signal averaging device and sent to an integrator which automatically computes the vitamer concentration from the observed peak areas[2]. There are three valves in the buffer stream, all of which must be controlled during the run. Actuation of the MSIS valve determines the time of sample inject; switching of the valve between the reservoirs determines the particular buffer to be used at a given time; and switching of the valve between the two columns determines whether the buffer stream passes through one column or two. Two buffers are necessary if pyridoxic acid concentrations are to be determined; otherwise, the system is isocratic. The two-column system is necessary to enhance the separation of some vitamers and the internal standard; the procedure and time program have been described elsewhere[2]. The time of sample inject, the start of the integrator as well as wavelength and other changes in the detector controls are synchronized and controlled by the microcomputer.

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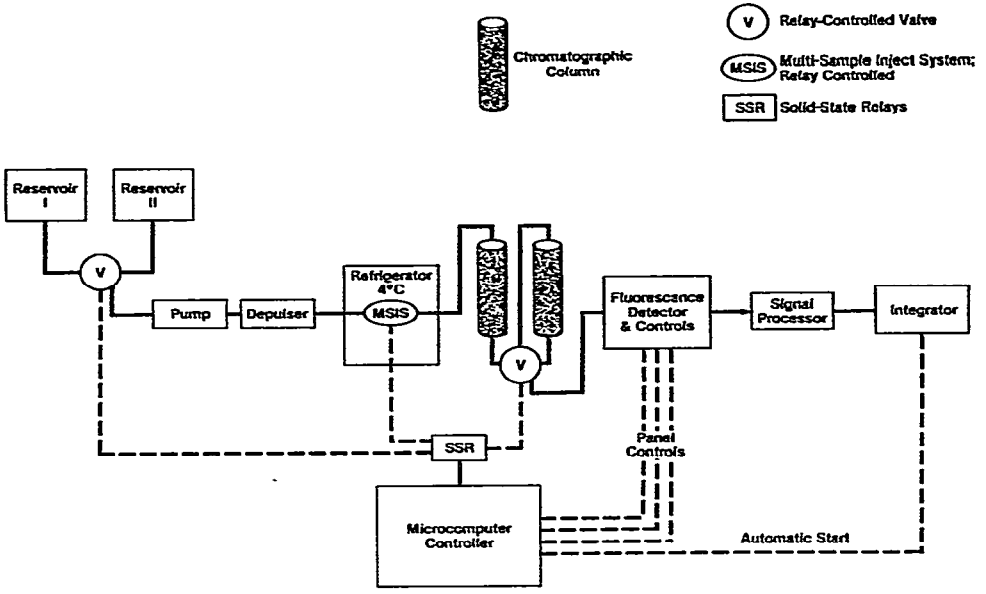

Fig. 1. Block diagram of automated chromatographic system. Dashed lines correspond to electrical leads from the microcomputer to various components.

Pump and depulsing system

A Milton-Roy minipump (Model 396, Laboratory Data Control, Riviera Beach, FL, U.S.A.) is used and is depulsed with two gauges and a needle valve[7]. When the sampling rate is increased, the depulsing system is modified as discussed later.

Multi-sample inject system and switching valve

The two switching valves shown are pneumatically activated (ACV-6-UHPa-HC; Valco, Houston, TX, U.S.A.) and are each controlled by a relay-activated solenoid valve (Model 062-4E1-36; Humphrey, Kalamazoo, MI, U.S.A.). The multi-sample inject system is composed of three parts: a twenty-sample loop rotary valve and a valve-drive unit (Models ROV-1-20 and VDU-20, Laboratory Data Control) together with another Valco valve (ACV-6-UHPa-HC). The drive unit can be either manually operated or automatically controlled by an external relay. The Valco valve has both a load and inject position. By incorporating this in combination with the rotary valve, all of the sample loops can be removed from the buffer stream when the Valco valve is in the load position. Thus, each of the sample loops can be loaded with the entire arrangement out of line. Sample loading is then possible while the rest of the entire system is processing an unknown.

Columns

In the present work, two water-jacketed columns are used, both packed with Aminex A-25 resin (Bio-Rad Labs., Richmond, CA, U.S.A.). The first is 20 cm × 6 mm and is maintained at 55°C, the second is 7 cm × 6 mm and maintained at 18°C.

Detector

A Perkin-Elmer fluorescence spectrophotometer (Model 650-40; Perkin-Elmer, Norwalk, CT, U.S.A.) with a 20-μl flow cell is used as the detector. Panel controls allow changes, for example, of excitation and emission wavelengths, signal amplification and photomultiplier gain to be implemented by a few key strokes. Key depression simply draws a small current to ground in the keyboard logic circuit which informs the internal microprocessor of the desired change in state. The existing spectrophotometer panel was modified by attaching leads from the microcomputer input/output buffer to the instrument key terminals. This allows an external microcomputer to observe and learn operator keyboard actions during manually controlled development runs. Once successful protocols are developed the microcomputer can simulate operator action for fully automatic multi-sample analysis.

Signal processor

The Oriel multifunction signal processor (Model 7600; Oriel, Stamford, CT, U.S.A.) takes the analogue signal from the fluorescence detector, converts it to a digital signal, performs a real time sliding average, and converts it back to an analogue signal which then goes to the integrator. This processor provides a wide range of response times (2 msec to 32 sec) and thus a "best" choice can be made for the signal-to-noise ratio in a particular application[8].

Integrator

A Chromatopac CR-1A (Model A221-16386-90; Shimadzu, Columbia, MD, U.S.A.) computing integrator is used to obtain the areas of observed peaks and hence, concentrations. In its automatic mode, it can be programmed to handle multiple internal standard weights and can be started by the external microcomputer employing the technique used with the detector.

Microcomputer control system

The microcomputer control system is a Rockwell AIM-65 with 4K bytes of memory (Rockwell, Anaheim, CA, U.S.A.). It is used to control all valves, integrator starts and the appropriate keys in the fluorescence spectrophotometer; the interface has been described in detail elsewhere[1]. The solenoid valves, which control the buffer switching valves, require high power (110 V a.c.) and are each activated by an optically isolated solid-state relay (Model 0AC5; OPTO 22, Anaheim, CA, U.S.A.). These relays are controlled by the microcomputer input–output buffer (or by manual switches in the development runs). The MSIS is controlled in a similar manner as the buffer-switching valves. The integrator is started by the microcomputer employing the technique used with the detector.

The system is unique in that it has a "learn" mode. In the "learn" mode, the microcomputer stores in memory a time-based record of manual operations performed on the instruments. In the "run" mode it simply instructs the instruments according to the operations "learned" by observing the analyst. Thus, one manual operation is sufficient to completely program the system, eliminating any need for further operator input.

Depulsing system for fast flow

The HPLC system has normally been run at 1.2 ml/min and a run to determine only the vitamer concentrations took approximately 75 min. If the flow-rate is increased to approximately 2 ml/min, the pressure was above 500 p.s.i.g. which suggested that a high-pressure steel system should be used. To avoid this, the depulsing system shown in Fig. 2 was devised. The flow is depulsed with three gauges and two needle valves. The first part of the system is made from stainless steel and the needle valves adjusted so that the pressure is only 300 p.s.i. when the buffer enters the remaining teflon tubing of the system. In addition the MSIS takes approximately 5 sec to change sample positions. During this time, the pressure rose precipitously at the higher flow rate. To avoid this, a bypass loop around the MSIS was introduced as shown in Fig. 2.

Sample handling and sensitivity

With the signal processor, it is possible to reduce the lower limits of detection for one of the important vitamers, PLP, to 0.1 ng/ml. The lower limits for the other vitamers are approximately 0.05 ng/ml. At these lower limits, it was found that sample handling became important. Under normal treatment, for example, when the concentration of pyridoxal phosphate falls below 1–2 ng/ml, a 50% loss of this vitamer occurs if the sample is kept for 1 h before injection and analysis. However, if degassed water is used in sample preparation and in the extraction procedure, the concentration remained stable for 24 h. In addition, the MSIS loaded with samples, regardless of vitamer concentration, must be refrigerated at 4°C to avoid loss over a 24-h period.

PROCEDURES AND RESULTS

Normally, one would have a clear idea of the instrument instructions and times for a given chromatographic system before automation is possible. This is so for the present system. To set the automatic program, the following procedure is employed. A solution of standards is loaded into the sample loop and the microcomputer is put into the "learn" mode. All initial panel controls and the proper position of valves are made. Injection is made and the integrator is started. During this development run, all entries of valves and functions on the panel and all valve state changes are per-

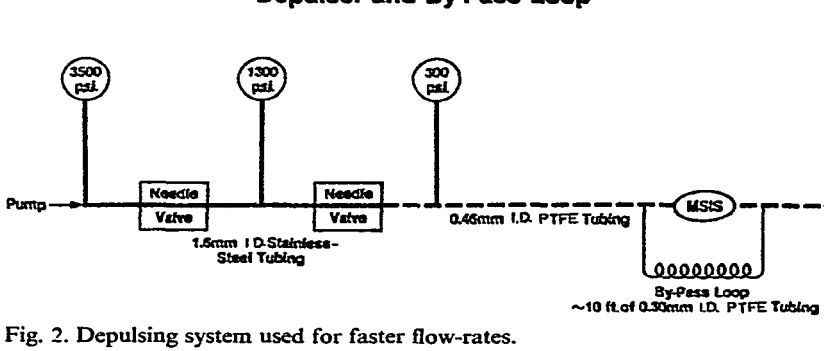

Fig. 2. Depulsing system used for faster flow-rates.

TABLE I

STABILITY OF VITAMERS IN SAMPLE LOOPS

Vitamer	*Concentrations (ng/ml)*		
	Sample 1 (0 h)	*Sample 19 (24 h)*	*Ratio sample 19 : sample*
PMP	26.5	26.3	0.99
PM	20.6	22.8	1.11
PNP	16.6	14.8	0.85
PN	28.1	30.8	1.09
PLP	32.7	35.0	1.07
PL	26.7	26.9	1.01
		Average	1.02

formed. This run can also serve as the calibration run for the integrator. At the end of the run, the microcomputer is given an "escape" command.

With the MSIS in the load position, nineteen samples are loaded into the loops. After each loading, the excess tubing is flushed with buffer. The twentieth loop must be reserved for final flushing with buffer. The nineteen different internal standard

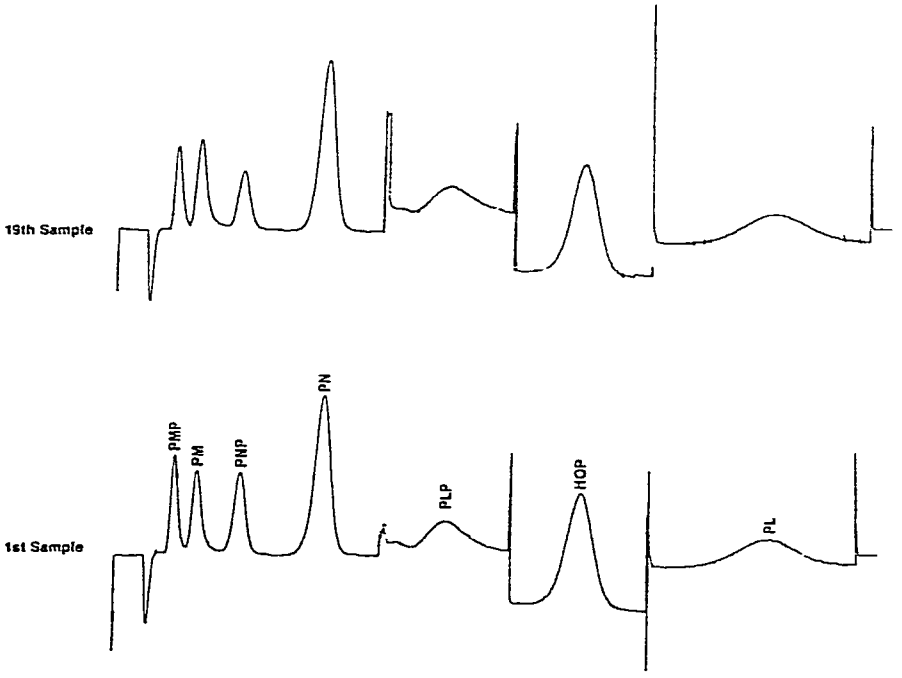

Fig. 3. Chromatographic traces for the first and nineteenth injection. The nineteenth sample was kept in the sample loop at 4°C for 24 h. The flow-rate was 1.2 ml/min and the concentrations of the different vitamers were approximately 25–30 ng/ml (see Table I).

weights are entered and stored in the integrator. The microcomputer is told that nineteen samples are to be run by entering the command to "RUN 19". The system then operates for approximately 24 h with minimal attention. With proper sample handling, no appreciable loss in vitamer content appears during this time (Table I). Fig. 3 shows the chromatographic traces for the same sample in loops 1 and 19 of the MSIS (pyridoxic acid was not included in this study as it is stable). Pyridoxine phosphate appears to be the most unstable with a loss of 15%; however, this particular form is rarely observed in samples.

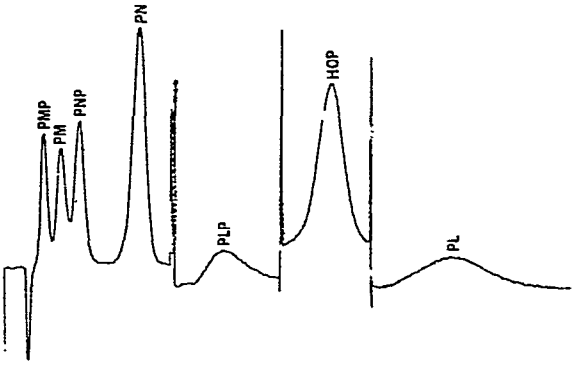

Fig. 4. Chromatographic traces where flow-rate was 2.1 ml/min. The concentrations were approximately 25–30 ng/ml.

One can increase the flow-rate and decrease the run times of the system if one so desires. There will be some loss of resolution as shown in Fig. 4 where the flow-rate has been increased to 2.1 ml/min as opposed to the normal 1.2 ml/min. The observed concentrations are still reproducible to within five percent for the partially overlapping peaks. For most samples that have been investigated, all forms of the vitamers do not appear and this partial overlap causes no problems. Thus, this higher-speed system is worthy of consideration.

CONCLUSIONS

The automation of the vitamin B_6 analyser system described here is simple and inexpensive and can easily be adapted with minor modifications to other HPLC applications. Sample through-put can be doubled with little effort but with some cost to resolution. However, for many real samples this presents no difficulty in the case of vitamin B_6. With proper sample handling, the system can reproducibly detect concentrations of the vitamers less than 0.1 ng/ml.

This example of automation illustrates several important points. First, a small inexpensive microcomputer can control a broad mixture of system components. Second, by simulating the keyboard operations of a typical instrument panel, the internal microprocessor logic and timing of individual instruments is by-passed. Finally, the use of a "learn" mode minimizes any chance of operation error after the initial development run.

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