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AUTOMATION OF PRE-COLUMN DERIVATIZATION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

APPLICATION TO ION-PAIR PARTITION CHROMATOGRAPHY

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SUMMARY

An automated pre-column derivatization system is described. Problems arising from the coupling of this AutoAnalyzer-type system with high-performance liquid chromatography are discussed in detail. The system has been applied to ion-pair partition chromatography. The ion-pairs of a mixture of some alkaloids (apoatropine, hyoscyamine, scopolamine and ergotamine) were formed with picric acid in an aqueous phase, extracted into chloroform and injected on-line on to a silica gel column for separation within 15 minutes. This study demonstrates the possibility of obtaining an automated pre-column derivatization system with a good relative standard deviation of $< 3\%$ per injection ($n = 6$). The linearity of the system for hyoscyamine and ergotamine is significant in the concentration ranges of 80–200 ng and 240–720 ng per injection, respectively. The experience gained is of general validity and could generate more studies in this area.

INTRODUCTION

Derivatization techniques in high-performance liquid chromatography (HPLC) are employed to increase the sensitivity or to improve selectivity. The derivatives can be made either before or after the chromatographic separation. The terms pre-column and post-column derivatization are used[1]. In post-column derivatization the eluent from the column is passed continuously into the reaction system, where the desired reaction occurs and the product is subsequently measured. The dead-volume in a reactor of this type, the diffusion processes and the influence of the mobile phase on the kinetics of the reaction may entail reduced resolution and a loss of sensitivity[2]. In pre-column derivatization the reaction is carried out first and the product is then injected on to the column. A series of studies on pre-column derivatization has been published and these have been reviewed by Ross[3]. If the number of samples to be analyzed is considerable, the advantages of pre-column derivatization may be limited especially when the reaction is tedious and poorly reproducible.

If ion-pair partition chromatography is to be employed, the substances must be injected in the form of ion-pairs[4]. The manual preparation of the ion-pair is relatively simple, but the extraction could be made quantitative only by treating the samples in a sophisticated manner. Assaying a large number of samples will therefore be expensive. The aim of this paper was to develop an automated pre-column derivatization system by coupling an AutoAnalyzer to the HPLC, as has been mentioned in a general way by Burns[5]. The problems which arise from this coupling are discussed.

EXPERIMENTAL

Reagents

The alkaloids examined were hyoscyamine, apoatropine, scopolamine and ergotamine (Sandoz, Basle, Switzerland). Picric acid and chloroform of analytical grade (E. Merck, Darmstadt, G.F.R.) were used for the formation of ion-pairs and for the extraction. The mobile phases, the chromatographic conditions and the loading of the columns with stationary phase are specified in the figures or in ref. 6.

Apparatus

The chromatographic system comprised a 1010 Hewlett-Packard chromatograph, a silica gel Si 100 column (100 × 4.6 mm) and a Perkin-Elmer LC 55 UV/VIS detector.

Fig. 1 is a diagram of the system employed for the formation of the derivatives. The peristaltic pump, fittings, mixing coils (2.6 mm I.D.) and the pump tubings were supplied by Technicon (Tarrytown, N.Y., U.S.A.). The system has been designed to provide optimum reaction conditions and for a sample volume of 4–5 ml in 0.1 *N* hydrochloric acid.

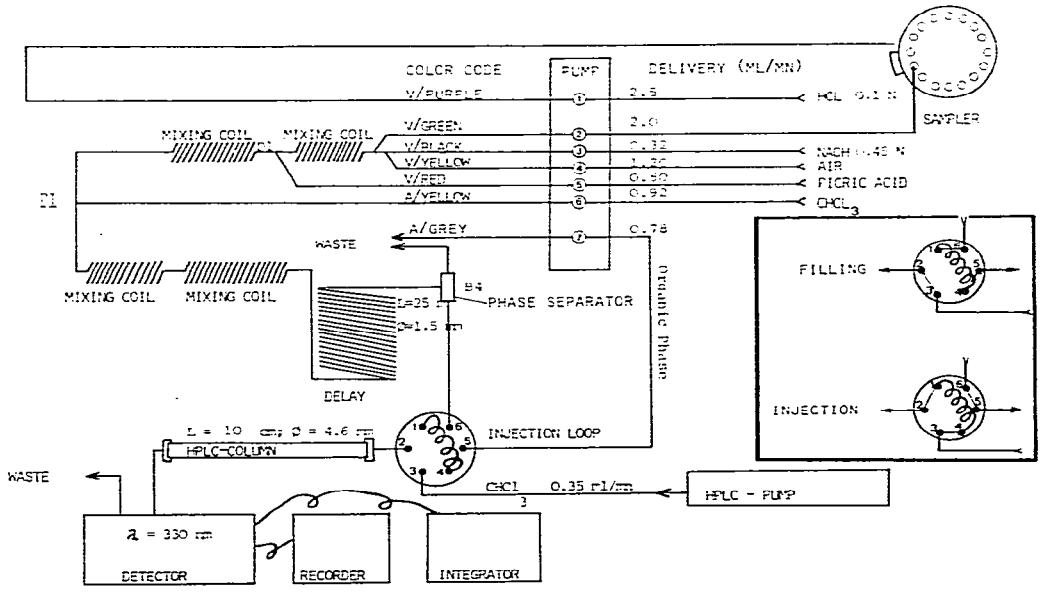

Fig. 1. Diagrammatic representation of the derivatization system.

The derivatization system can be integrated with the HPLC as shown in Fig. 2. The coupling of the sampler (CS 40 Automatic Sampler; ChemLab Instruments, Ilford, Great Britain) and of the loop injector (Rheodyne 7010-A, Berkeley, Calif., U.S.A.) is achieved by means of a programming unit developed in our department. The volume injected is 100 μl in each case.

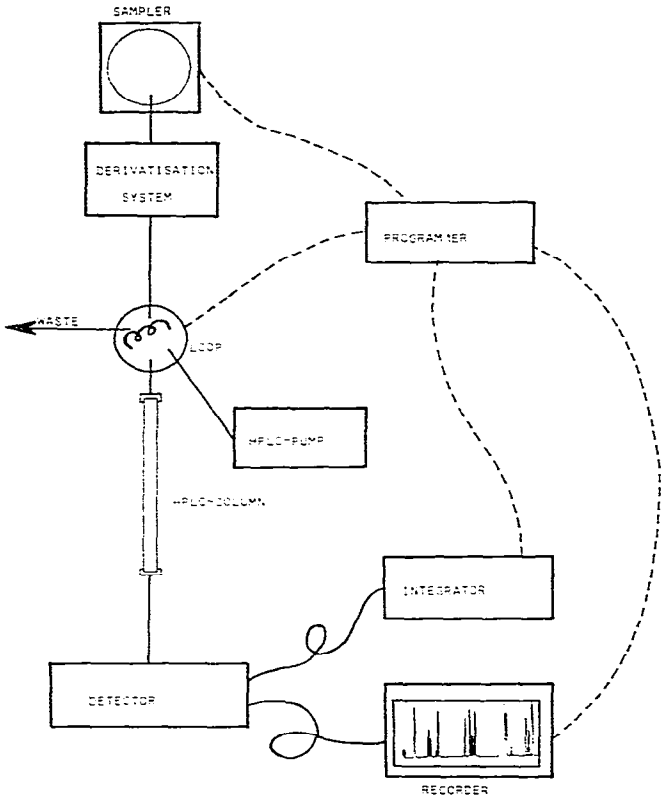

Fig. 2. Integration of the derivatization system with the HPLC instrument.

RESULTS AND DISCUSSION

Ion-pair partition chromatography

For ion-pair partition chromatography the alkaloids must be injected in the form of ion-pairs, *e.g.*, with picric acid. This is achieved by treating hyoscyamine, apoatropine, scopolamine and ergotamine with picric acid in the aqueous phase. The resulting ion-pairs are extracted with chloroform and injected on to a silica gel column which has been charged with a buffered picric acid solution[6]. Complete separation is achieved within 15 min (see Fig. 5).

General aspects of automated analysis

In automated analysis the reaction time depends not only on the reaction kinetics but also on the dead-volume and the diffusion processes in the derivatization

system. In consequence, complete conversion into the desired reaction product may be delayed and the period required for analysis correspondingly extended. If, for example, the organic phase is allowed to flow continuously through a flow cell in a photometer, then the function shown in Fig. 3 is obtained when the reaction product is passed through the cell. It is necessary to distinguish between the "wash-in", *i.e.*, the time that is required for the reaction to proceed to the limit (the attainment of the steady state), and the "wash-out" denoting the time that elapses until the base line is reached. This must be selected so as to be sufficiently large to preclude interference between the samples. The "delay time" denotes the time that elapses from the moment when the sample is introduced into the derivatization system to the moment when it reaches the sample loop, and the synchronization must take account of this.

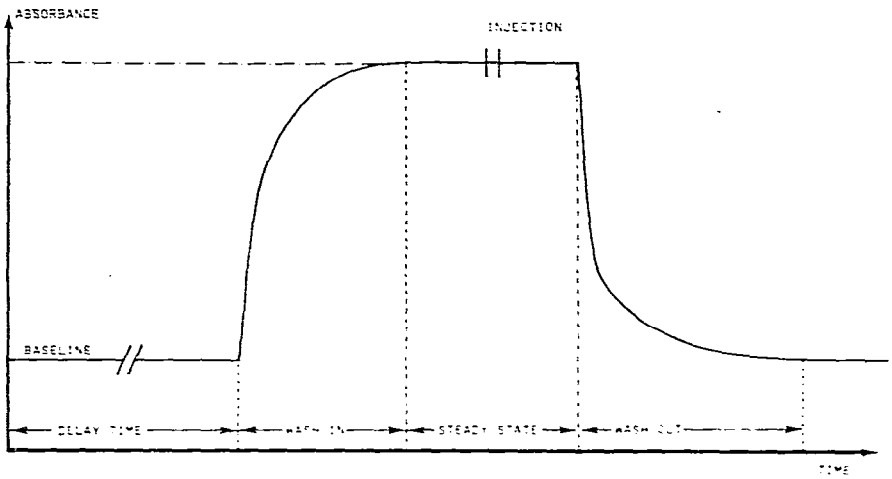

Fig. 3. Concentration characteristics of the continuous analysis.

Coupling with HPLC

Coupling with HPLC is carried out as specified in Fig. 1. For this purpose the sample solutions to be examined are converted into the reaction product at specified times, one after the other, and these are injected via the sample loop on to the HPLC column. The timing required for this coupling of the sampler and the loop can be deduced from the scheme shown in Fig. 4.

When the sampling needle is dipped into the first sample (aqueous) the loop is adjusted to the filling position. After sampling, the needle is placed in the washing vessel and at the same time the organic phase in the loop is injected on to the column. For the first injection, only the blank of the derivatization system is injected on to the column. When the extracted ion-pairs reach the loop, the second sample is drawn into the derivatization system and the sample loop is simultaneously charged with the reaction products. After the second sample has been taken the loop is turned and the ion-pairs are injected on to the column. This procedure is then repeated until all the samples have been analyzed.

In order to ensure that the derivatization system is synchronized with the HPLC, the delay time must correspond to the time required for chromatography.

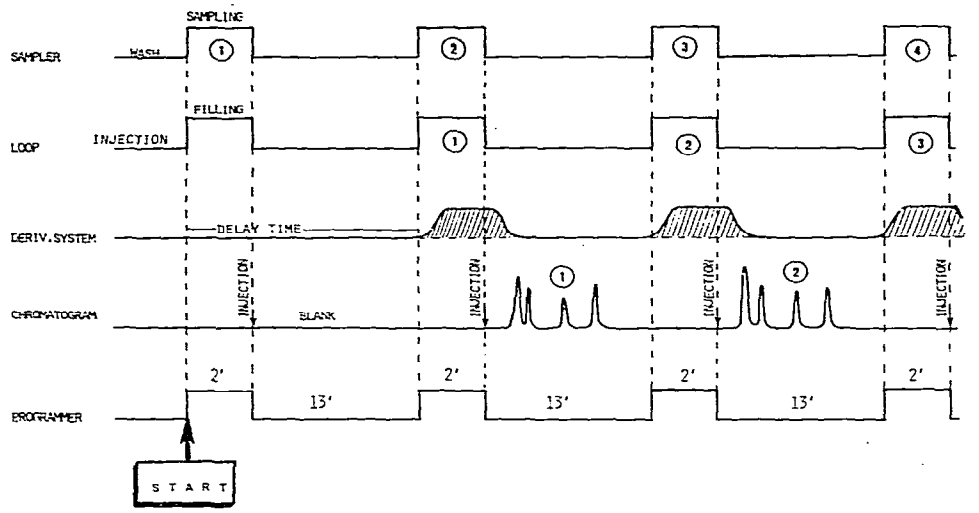

Fig. 4. Scheme of the automatic pre-column derivatization.

Since the conversion of alkaloids into the corresponding ion-pairs and their extraction occurs rapidly, it has been necessary to use a delay coil into the derivatization system, to compensate for this effect (Fig. 1).

The time required for sampling must be selected so that the reaction product has undergone as nearly as possible complete reaction. In practice we found that the 90% level of the steady state is achieved within 60 sec. This sampling time is sufficient for good reproducibility[2], but because of the deviations in the pumping rates of the pump tubing a sampling time of 2 min is chosen.

Reproducibility

The overall standard deviation of the pre-column derivatization is given by the expression:

$$s_{\text{total}} = \sqrt{s^2_{\text{HPLC}} + s^2_{\text{Deriv.}}}$$

The experiments showed that the reproducibility of the derivatization system ($s_{\text{Deriv.}}$) and the HPLC (s_{HPLC}) is $< 2\%$ so that, when the whole system is working properly, the overall standard deviation of the system may be expected to be $< 3\%$. To verify this calculation, a mixture of alkaloids was repeatedly injected and the chromatograms shown in Fig. 5 were obtained. The evaluation of the chromatograms indicates a relative standard deviation ($n = 6$) of 1.5% for ergotamine, 1.2% for hyoscyamine and 2.4% for apoatropine. Scopolamine is separated but it cannot be determined because of the small chromatographic peak. Pre-column derivatization can thus be automated and it makes possible sound analytical work.

Linearity

The linearity of the system was investigated for hyoscyamine and ergotamine in concentrations ranging from 80 ng to 200 ng and from 240 ng to 720 ng per injection, respectively. Table I shows that the regressions are linear.

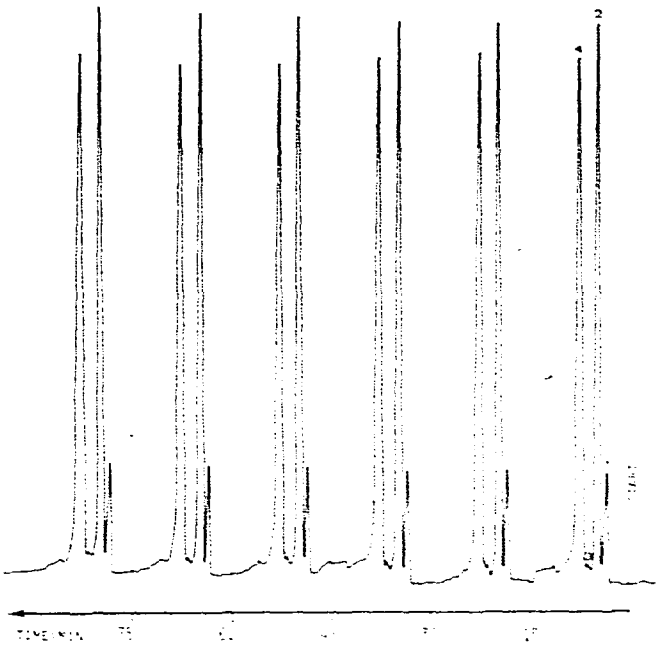

Fig. 5. Reproducibility of the automatic pre-column derivatization. Column (10 cm × 4.6 mm I.D.): silica gel, charged with picric acid, LiChrosorb Si 100 (Merck). Mobile phase: chloroform saturated with picric acid, flow-rate = 0.35 ml min. Detection: 330 nm (Perkin-Elmer LC-55). Injection: 0.2 μg of apoatropine (1), 0.2 μg of scopolamine (3), 2.0 μg of hyoscyamine (2) and 6.0 μg of ergotamine (4); volume injected = 100 μl (Rheodyne loop).

TABLE I

LINEARITY CHECK OF HYOSCAMINE AND ERGOTAMINE

Amount of hyoscyamine per injection (ng)	*Peak height, $\bar{X}$ (2)*	*Amount of ergotamine per injection (ng)*	*Peak height, $\bar{X}$ (two injections)*
80	88.0	240	74.0
120	121.5	360	114.0
160	170.0	480	151.0
200	213.0	600	193.0
		720	230.5
$r = 0.9988$		$r = 0.9999$	

Application

As mentioned at the outset, one of the motivating factors for employing derivatization before or after the chromatographic column is the attainment of greater sensitivity. This is necessary if very small concentrations of the substance are to be determined. This case arises for example in the determination of the dissolution rate of dosage forms containing a very small amount of a drug. Bellergal Retard® tablets provide a very good example of this because they contain in addition to phenobarbital a mixture of ergotamine tartrate (0.6 mg) and hyoscyamine (0.2 mg).

The dissolution rate for hyoscyamine and ergotamine from a Bellergal Retard tablet in 100 ml of artificial gastric juice was tested according to the method in ref. 7. After specified intervals of time, a sample (5 ml) was taken and analyzed and the recording shown in Fig. 6 was obtained. Besides the sensitivity, we also observe a certain selectivity of this ion-pair partition method because of the absence of any phenobarbital peak.

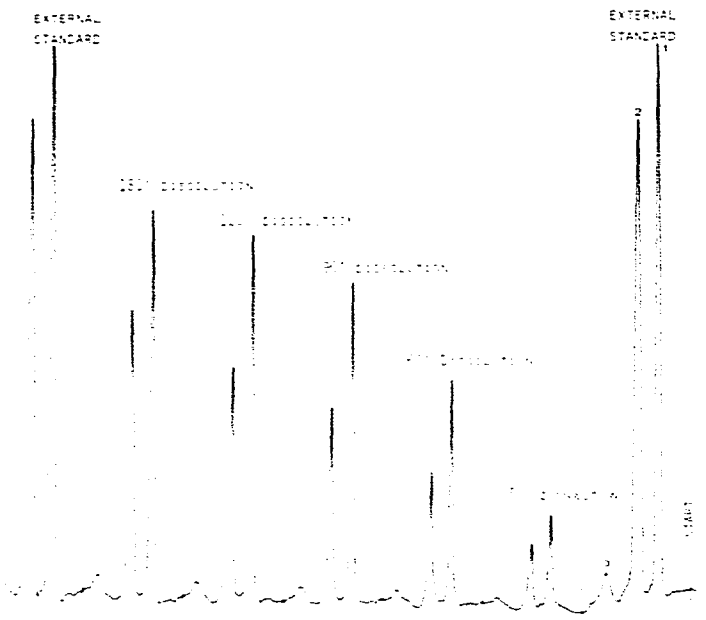

Fig. 6. Recording of the determination of the dissolution rate of hyoscyamine (1) and ergotamine (2) from a Bellergal Retard tablet (3 unknown). External standard: 0.2 μg of hyoscyamine, 0.6 μg of ergotamine. For example, after 30 min dissolution, the amounts injected are 80 ng ergotamine and 32 ng hyoscyamine. Chromatographic conditions as in Fig. 5.

CONCLUSIONS

The results show that the pre-column derivatization can be automated by coupling a derivatization system with the HPLC injection system. In this example, the volume of the sample that is required can be reduced by miniaturizing the derivatization system to the appropriate degree, *e.g.*, by employing the AutoAnalyzer systems of the second or third generation. Since the system shows good reproducibility, it can be used for the determination of large number of samples. The system could easily be adapted for other pre-column derivatizations by means of appropriate modification of the manifold.

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