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LIQUID COLUMN SWITCHING EXTRACTION AND CHROMATOGRAPHY FOR PROGRAMMED FLOW PREPARATION

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SUMMARY

A system for programmed flow preparation was developed, converting the usual batch process into flow manipulations. All processes in preparative chemistry, including reactions, extraction and chromatography, can be performed in all-glass or PTFE tubing by passing the solution along a programmed course controlled by simple valve operations. As an application of the system to chemical synthesis, acylation reactions such as acetylation of the hydroxyl group and protection of α-amino acids were carried out successfully. To maintain high efficiency and reproducibility of the system, the switching mechanism of the extraction process was examined and the results were applied to the quantification of plant components such as curcumin of *Curcuma longa* as a model experiment. Although the system combines almost all of the preparation techniques conventionally used in analytical and synthetic chemistry, droplet current and chromatographic liquid–liquid extraction techniques assured continuous processing and provided a high clean-up efficiency before chromatographic separation.

INTRODUCTION

Many organic preparations involve, for example, synthesis, isolation of natural products and derivatization prior to analytical study to permit the quantification of biological components, which in many instances have been carried out in the laboratory with manual batch operations including funnel-to-funnel transfer of solutions. These operations may be very tedious, time consuming and hazardous. Various types of continuous procedures for combining extraction and chromatography with automation of the system have been considered[1–3]. However, a flexible and universal flow system applicable to a wide range of preparations has not yet been established. In our laboratory, a direct fractionation procedure using simple liquid chromatography has been developed for preparative procedures in organic synthesis[4–9]. In continuation of our work, the incorporation of the preliminary extraction process into the system will be examined in detail in order to improve the efficiency of the system.

"Liquid column switching extraction and chromatography for programmed flow preparation" may afford an improved preparation system without the defects characteristic of former procedures using batch processing. This new system is so

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designed that the liquid contents flow through interconnected columns and tubes fitted with stoppers and switching valves, permitting programmed control of the flow.

A preliminary fractionation is generally required for isolation of a pure constituent from a complex mixture. This procedure ("clean-up") is accepted as an indispensable pre-treatment, especially in the analysis of biological fluids. The fractionated sample is finally injected into a chromatographic column for resolution of the sample mixture.

Binary aqueous organic solvent liquid–liquid extraction is commonly adopted for the primary fractionation procedure. Batch processing, including extraction in a separating funnel, followed by washing with water, drying and evaporation procedures are commonly used techniques in the chemical laboratory. Several liquid–liquid distribution procedures seemed to be practical for extending procedures used in batch methods to continuous flow systems. Droplet counter-current extraction is considered to be one of the simplest useful procedures. It is possible to control the distribution coefficient of compounds in the aqueous organic solvent phase by adding inorganic salts to the aqueous phase and adjusting the pH, allowing easy fractionation of acids, bases and neutral compounds by applying the droplet counter-current extraction. In this instance, improving the column efficiency by increasing the number of theoretical plates is not considered. Extraction chromatography, on the other hand, can be used instead of droplet counter-current extraction. Thus binary aqueous–organic partition chromatography using a packed column containing an aqueous stationary phase on the surface of the packing material seemed acceptable for our needs.

These two extraction procedures were examined in order to construct a programmed flow preparation system including a sequencer. A reactor, a dryer for eliminating water from the extraction solvent, a flow-type evaporator and a pressure converter which is required to connect the high-pressure components of the extraction process with the low-pressure components of the evaporation process were specially designed.

In this paper, the design of the extraction–chromatography switching system and its application to synthetic preparation and natural product analysis are described.

EXPERIMENTAL

Construction materials

Glass tubes. The following sizes of CIG glass tubing (Kusano, Tokyo, Japan) were used: (A) 6 cm × 4 mm I.D.; (B) 15 cm × 5 mm I.D.; (C) 10 cm × 1 cm I.D.; (D) 15 cm × 1 cm I.D.; (E) 30 cm × 1 cm I.D.; (F) 10 cm × 1.5 cm I.D.; (G) 30 cm × 1.5 cm I.D.; and (H) 30 cm × 2.2 cm I.D.

PTFE tube. Connection between the glass tubes and other interfaces was achieved using PTFE tubing of I.D. 1 mm for preparative works and 0.5 mm for analytical works. The column stoppers were also made of PTFE (Kusano).

Valve. Three-channel PTFE valves were inserted between the reactor, aqueous columns, dehydrator, pressure converter and evaporator, as illustrated in Fig. 1. The reactor and chromatograph column were equipped with eight-channel valves.

Diatomaceous earth extraction column. Commercially available diatomaceous

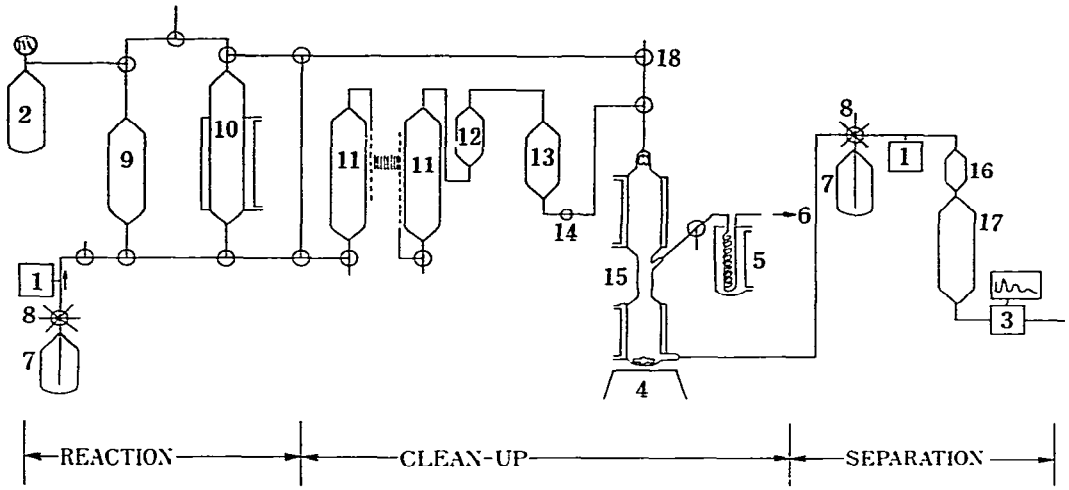


Fig. 1. Minimal flow preparation system. 1 = Pump; 2 = argon cylinder; 3 = detector; 4 = magnetic stirrer; 5 = condenser; 6 = aspirator; 7 = solvent flask; 8 = eight-channel valve; 9 = injector; 10 = reactor; 11 = aqueous column; 12 = dryer; 13 = pressure converter; 14 = needle valve; 15 = evaporator; 16 = pre-column; 17 = separation column; 18 = three-channel valve. All of these interfaces are connected with PTFE tubing and column stoppers suitable for pressures of up to 50 kg/cm^2.

earth (Extrelut; Merck, Darmstadt, G.F.R.) was crushed into granules and packed in a glass tube (size B) by the slurry packing technique. The column was injected with water, which was pre-saturated with diethyl ether.

Chromatography

The pre-columns and separation columns were packed with silica gel (Wako-Gel LC-10; Wako, Osaka, Japan). The effluent was monitored with a refractive index (RI) detector (R-401; Waters, Milford, MA, U.S.A.) or a UV detector (UVILOG II; Kusano) connected with a recorder.

Samples and chemicals

All chemicals used were of reagent grade and supplied by Wako. Rhizoma of *Curcuma longa* was kindly supplied by Professor H. Itokawa.

Acetylation of testosterone

Testosterone (200 mg), pyridine (1 ml) and acetic anhydride (1 ml) were placed in a glass tube reactor (size D) and the solution was allowed to stand for 3 h. Methanol (1 ml) was added to the solution, which was allowed to stand for a further 30 min. Ethyl acetate was injected into the bottom of the tube at a flow-rate of 10 ml/min, and the effluent was then injected into a glass tube (size E), which contained 5% sodium carbonate solution (18 ml), using a switching valve. Acetic acid was removed into the basic aqueous phase and the effluent was passed into 5% hydrochloric acid (18 ml) in a glass tube (size E) to eliminate pyridine. The organic layer was then pumped through a tube (size D) containing saturated sodium chloride solution (9 ml) to remove most of the moisture before the organic layer was dried by passing through a tube (size C) containing sodium sulphate. The dry organic layer thus obtained was then passed to tube C. A maximal pressure of 5 kg/cm^2 was maintained using an automated pressure

gauge, and the outlet was connected to a vacuum evaporator. The solution in the evaporator was concentrated at the bottom where the outlet was connected to the chromatographic system, using another pump and a switching valve which allowed selection of the correct mobile phase in order to perform a particular analysis. The resulting residue in the evaporator was dissolved in ethyl acetate–*n*-hexane (20:80) (3 ml) and the solution was injected into a silica gel column (size E) through a short pre-column (size C). Two washings of the inner face of the evaporator using the same solvent allowed complete sample injection, and the valve switching allowed continuous flow operation. Monitoring of the effluent by the RI detector made it possible to collect easily the desired product fraction according to the peak of testosterone acetate; the yield was 220 mg (96%).

Protection of α-amino acids

α-Amino acid (0.05 mole) and 2 *N* sodium hydroxide solution (25 ml) were placed in a reactor tube (H), the surface of which was cooled by the circulation of ice-water. The solution was stirred by bubbling argon through the tube. A solution of benzyloxycarbonyl chloride (10 ml) in diethyl ether (10 ml) was injected with continuous stirring and then 4 *N* sodium hydroxide solution (19 ml) was added slowly at a flow-rate of 1 ml/min. After the addition of alkaline solution, the contents were further stirred at room temperature for 2 h. The passage of argon was stopped and diethyl ether (200 ml) was injected as droplets at a flow-rate of 5.6 ml/min to remove any ether-soluble materials. The ethereal layer was collected from the drain at the top of the tube, and the layer remaining in the tube was pushed out by injection of carbon tetrachloride into the bottom of the tube. The carbon tetrachloride used was recovered from the bottom and the contents were again stirred by bubbling in argon. Ethyl acetate (20 ml) and 10% hydrochloric acid (30 ml) were injected. After 3 min, the passage of argon was stopped and ethyl acetate was injected continuously as droplets at a flow-rate of 10 ml/min. The effluent was switched to a column (size E) containing saturated sodium chloride solution (18 ml) and then to a dehydration column (size F) containing anhydrous sodium sulphate. The dried effluent was introduced into the pressure converter and the evaporator consecutively. The residue in the evaporator was used in the next synthetic stage with no further purification.

N-Benzyloxycarbonyl amino acid (Z-AA) obtained as above (0.046 mole) and *p*-nitrophenol (6.4 g) were placed in a reactor tube (size H) equipped with a PTFE filter at the bottom neck of the tube. The contents were dissolved in absolute ethyl acetate (60 ml) and the resulting solution was cooled with ice-water circulation and stirred by bubbling in argon. A solution of dicyclohexylcarbodiimide (DCC) (9.5 g) in ethyl acetate (20 ml) was injected. After 30 min, the cooling was stopped and stirring at room temperature was continued for 1 h. Argon injection was switched to the top of the tube, the resulting precipitates were filtered off through the filter and the filtrate was forced into the evaporator through the pressure converter. The residue collected at the bottom of the evaporator was dissolved in benzene, giving a total of 50 ml and a 5-ml aliquot of the benzene solution was injected into a silica gel pre-column (size C) connected with a separation column (size G). The solvent was switched to ethyl acetate–*n*-hexane (10:90) for the *p*-nitrophenyl ester of Z-Leu (Z-Leu-ON*p*) and ethyl acetate–benzene (2:98) for Z-Phe-ON*p*, and the effluent was monitored by an RI detector to obtain the desired fraction of the product; the yield was 68% for Z-Leu-ON*p* and 72% for Z-Phe-ON*p*.

Flow extraction and separation switching system for methanol extract of Curcuma longa

The outlet of the diatomaceous earth extraction column was connected with a UV detector set at 254 nm, the outlet of which was connected with another column (size A) packed with glass beads (60 mesh). A methanol extract of rhizoma of *Curcuma longa* was prepared by soaking in methanol (4 ml/g) for 1 week. An aliquot of 1 μl of the extract was injected on-column and then extracted with diethyl ether at a flow-rate of 0.8 ml/min. Ether-soluble materials were quickly eluted from the column and concentrated in the bead column evaporator within 2 min. Completion of the extraction occurred when the UV spectral curve merged with the baseline. After the inner pressure of the evaporator had returned to 1 atm, the pump was connected with the evaporator directly and the outlet of the evaporator was switched to the separation column (size B) packed with silica gel. The outlet of the column was connected with the UV detector. Then diethyl ether was injected into the evaporator at a flow-rate of 0.8 ml/min, flushing out the air through the valve between the evaporator and the separation column, before the ethereal solution was switched to the separation column by appropriate valve operation. The effluent was monitored by UV detection to give the final chromatogram.

RESULTS AND DISCUSSION

Lipophilic compounds are commonly involved in the separation of synthetic reaction products, natural products or biological constituents. They are usually isolated by binary liquid–liquid extraction using an aqueous organic solvent such as diethyl ether, ethyl acetate or chloroform. As an aqueous solvent extraction process, droplet counter-current distribution seems the most suitable for applying batch extraction procedures to continuous flow systems. Liquid–liquid distribution techniques allow large samples to be loaded for application to the flow preparative fractionation system.

On the other hand, in analytical work, quantitative extraction of the compound of interest is indispensable even in the preliminary clean-up process. A chromatographic system consisting of a water-coated support and an organic solvent such as diethyl ether seemed preferable for high-efficiency extraction. Aqueous organic solvent extraction and enrichment of the sample by using an open-bed column packed with diatomaceous earth or cellulose has been widely used as for clean-up. To incorporate such a system into the flow system and to increase the column efficiency by minimizing the theoretical plate height, a closed-bed column, packed with diatomaceous earth coated with water, and using diethyl ether as the eluent was examined.

Construction of the system

The essential components of the programmed flow preparation system are (a) a reactor for synthetic work or for sample derivatization in the analysis of biological specimens; (b) an extractor for preliminary clean-up of the mixtures and an evaporator to concentrate the solution; and (c) chromatographic apparatus for final separation. All of the required sequences are performed consecutively throughout the flow system by simple valve operations controlling the column-to-column switching. Based on the concept described above, a preparative flow system was designed using

glass and PTFE tubes so that it will withstand most chemicals. Fig. 1 shows schematically the minimal fow preparation system. The applications of this system to a general synthetic reaction and for the analysis of plant extracts by using an analogous system of smaller capacity is discussed below.

Acetylation of testosterone

Acylation is one of the most frequently used reactions in preparative chemistry. We first applied the system to the acetylation of testosterone, which can be carried out with simple transformation of the usual batch process into flow procedures. The entire process of this reaction is shown schematically in Fig. 2. The illustrations in each boxes indicate the processes that take place in the corresponding interfaces in Fig. 3. Also in Fig. 3, the course along which the solute passes is indicated by the bold lines.

After the reaction had proceeded to completion, acetic anhydride was decomposed with methanol and the extraction solvent was injected into the reactor. The

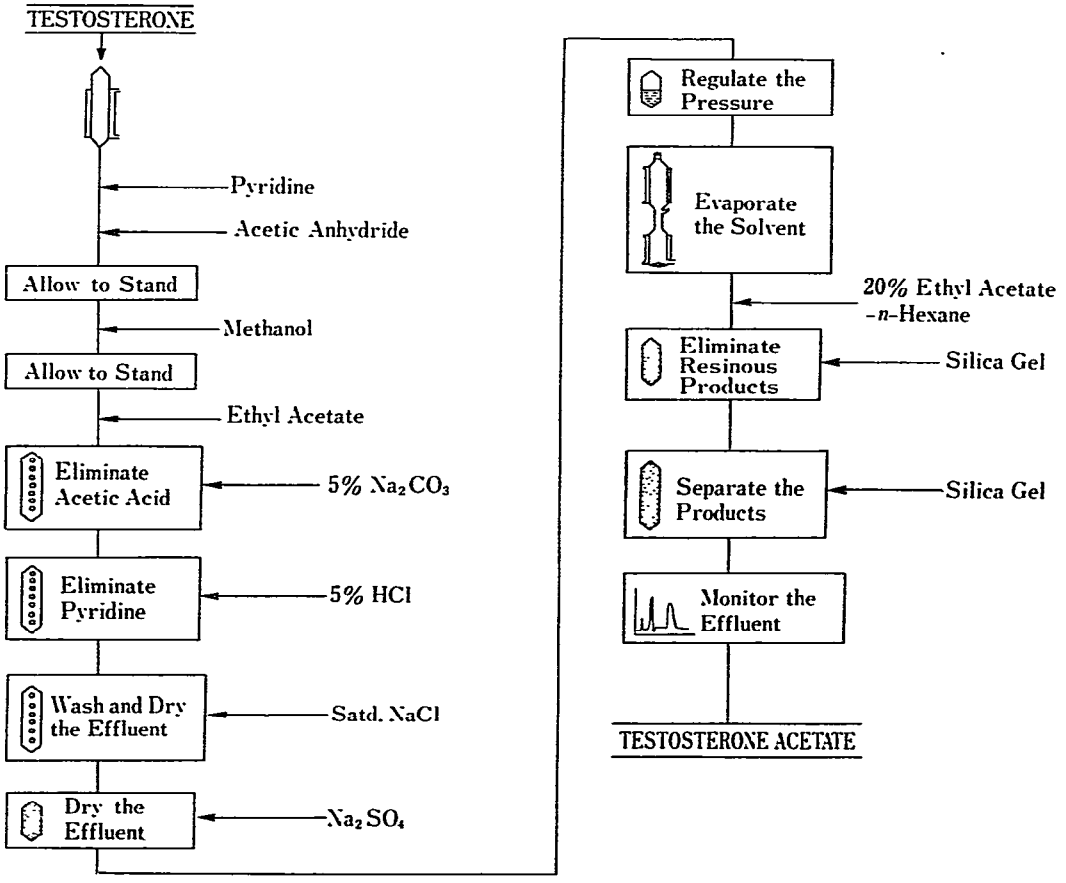


Fig. 2. Flow chart for preparation of testosterone acetate from testosterone. Arrows show the addition of the materials indicated. Insets show each procedure and interfaces used.

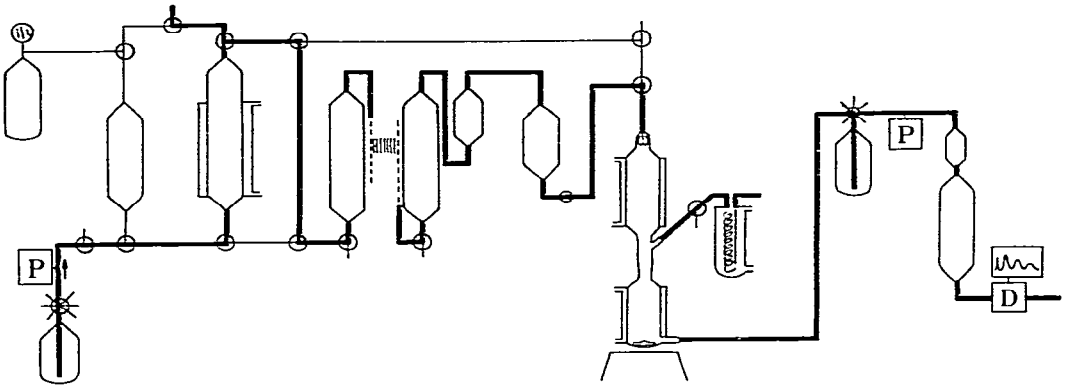


Fig. 3. Flow diagram for preparation of testosterone acetate.

$$\text{Testosterone} \xrightarrow[\text{Pyridine}]{\text{Acetic anhydride}} \text{Testosterone acetate}$$

The bold line shows the flow course followed during the operation. Starting material and reagents were injected by the three-channel valves at the top of the reactor. The extraction solvent was injected by the pump on the left-hand side. Acidic and basic waste fluids were recovered via the three-channel valves at the bottom of the aqueous columns.

product and excess of reagent were introduced into the first extraction column containing aqueous sodium carbonate solution to remove acetic acid. In the second extraction column, into which diluted hydrochloric acid was pre-injected, the droplets were washed to remove pyridine. Then the organic phase was introduced into the liquid column containing saturated sodium chloride solution, by means of which the organic phase could be dehydrated. Complete dehydration was achieved in the next stage by passing the effluent through the column packed with dehydrating agent.

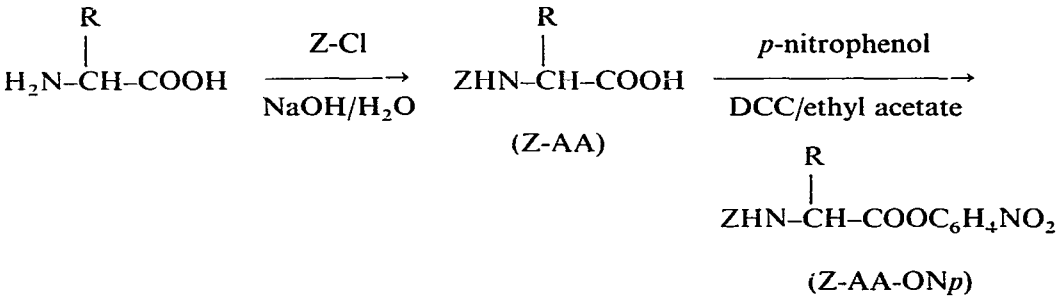
The organic extract thus obtained was then continuously introduced into the small empty glass tube. This interface is like a sophisticated pressure converter controlled by a pressure gauge connected to the pump and by a needle valve connected to the evaporator. Thus the high-pressure extraction components and low-pressure evaporation components were readily switched under the continuous flow control. The flow-rates before and after the pressure converter were controlled so that the converter fulfilled its function when solvent was present in it. It is unfortunate that the most suitable chromatographic solvent is often ill-suited for extraction, and *vice versa*, so elimination of the solvent before chromatographic separation is indispensable. Therefore, the extract in the converter was forced into the evaporator from the bottom through the needle valve by vacuum aspiration. In the evaporator, the solute was concentrated at the bottom because it was so designed that the solvent flows from top to bottom along the inner ground-glass surface.

The residue in the evaporator was redissolved in the solvent for chromatography and the solution was injected first into the pre-column by the pump inserted between the eight-channel valve and the pre-column. This valve is necessary for switching to select the correct mobile phase for a particular separation. The effluent

from the separation column was monitored with an RI detector and the chromatogram obtained is shown in Fig. 4. The time needed for the clean-up procedure and the separation was about 50 min, which is about three times less than in the usual batch process. The yield of the product was very high and reproducible.

Protection of α-amino acids

Recent interest in the preparation of bio-oligomers led us to examine the application of the flow system to α-amino acids, and the full protection of leucine and phenylalanine was investigated. A two-step sequence was required for this purpose, namely, benzyloxycarbonylation of the primary amino group and a subsequent condensation reaction of the carboxyl group with *p*-nitrophenol:



The first step was performed by the usual Schotten–Baumann procedure using a reactor tube in which the solution can be vigorously stirred by bubbling in argon and cooled with ice-water circulating in a jacket around the tube. After washing the mixture to remove neutral materials, the pH was adjusted to liberate the product, which can be extracted with organic solvent. The extract was dehydrated and concentrated to obtain crude Z-AA with minimal carry-over loss during the clean-up. The products were used directly in the next synthetic stage after transfer to a reactor tube with the solvent of the next reaction.

Condensation of Z-AA with *p*-nitrophenol was performed with DCC whilst stirring with argon. The resulting mixture contained the reagent-derived precipitates that were filtered off by passing through the PTFE filter on the column stopper at the bottom of the tube. The filtrate was evaporated and purified by silica gel chromatography.

All of these procedures were achieved on-line in the closed system so that the product decomposition was minimized. The chromatograms obtained are shown in Figs. 5 and 6 for Z-Leu-ON*p* and Z-Phe-ON*p*, respectively. Excess of reagent and side-products are indicated by the arrows.

Flow extraction and separation of plant components

Finally, we applied the system to the analysis of natural specimens. In this case, the system used for synthesis was reduced to a small-scale model. An extraction column was examined in detail and the packed partition column described under Experimental was developed. Also, evaporation was carried out in a short glass tube

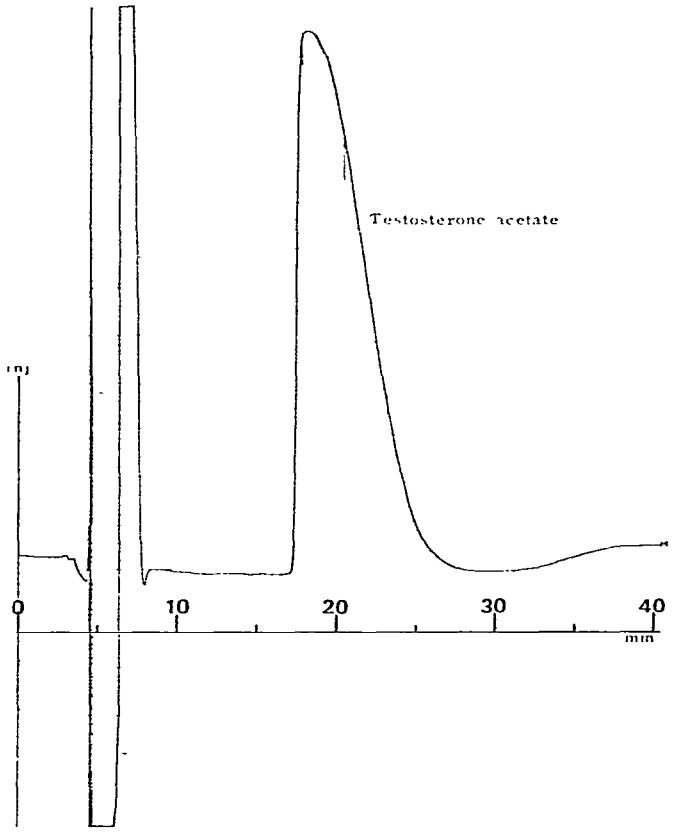


Fig. 4. Chromatogram of testosterone acetate effluent from the system, monitored with an RI detector. Flow-rate, 3 ml/min; mobile phase, ethyl acetate–*n*-hexane (20:80).

packed with glass beads. All of these interfaces were arranged in almost the sequence illustrated in Fig. 1, and a small amount of sample solution was injected into the extraction column by the on-column method.

Ether-soluble components were extracted with diethyl ether as the organic mobile phase and the effluent from the column was monitored with a UV detector. The effluent from the detector was concentrated by passing it through the evaporator, in which the most of the solvent was evaporated. When the detector signalled that a solute peak had emerged, time-delayed operation of the three-channel valve permitted the concentrated effluent containing the solute to flow into the separation column packed with silica gel using diethyl ether as the mobile phase. Of course, the other solvent system can be selected in this stage if necessary. Thus two-dimentional extraction–separation chromatography was achieved continuously, as illustrated in the chromatogram shown in Fig. 7.

The results show clearly that the flow preparation system can be applied to a wide range of analyses of biological specimens such as urine and serum, which are under investigation in our laboratory using the same system equipped with a column-type reactor for pre- and post-column derivatization.

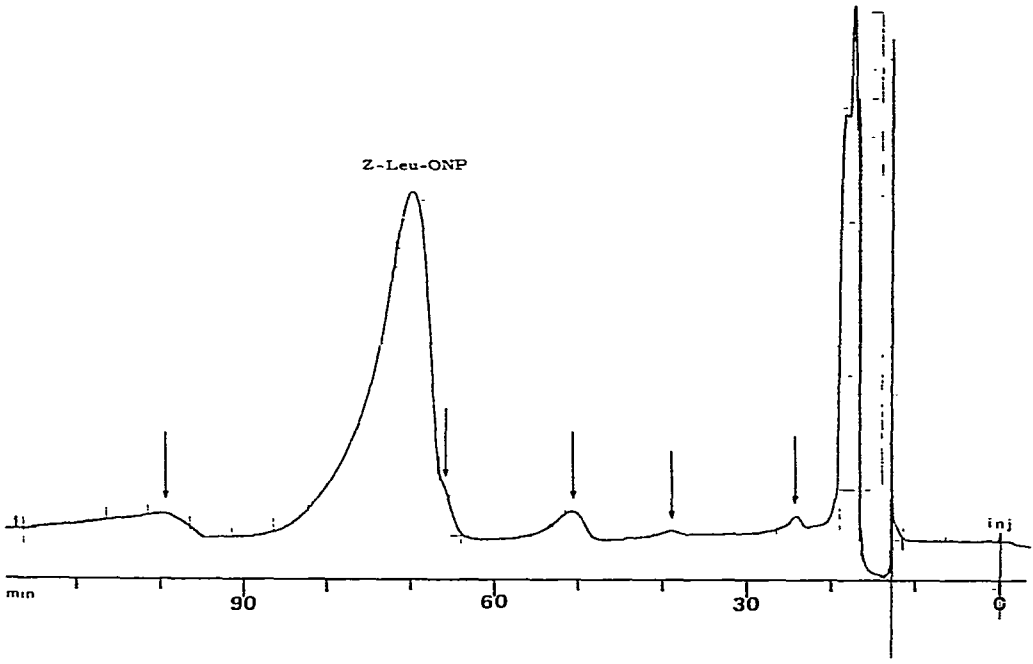


Fig. 5. Chromatogram of Z-Leu-ON*p* effluent from the system, monitored with an RI detector. Flow-rate, 4 ml/min; mobile phase, ethyl acetate–*n*-hexane (10:90). The arrows show the side-products and excess reagents.

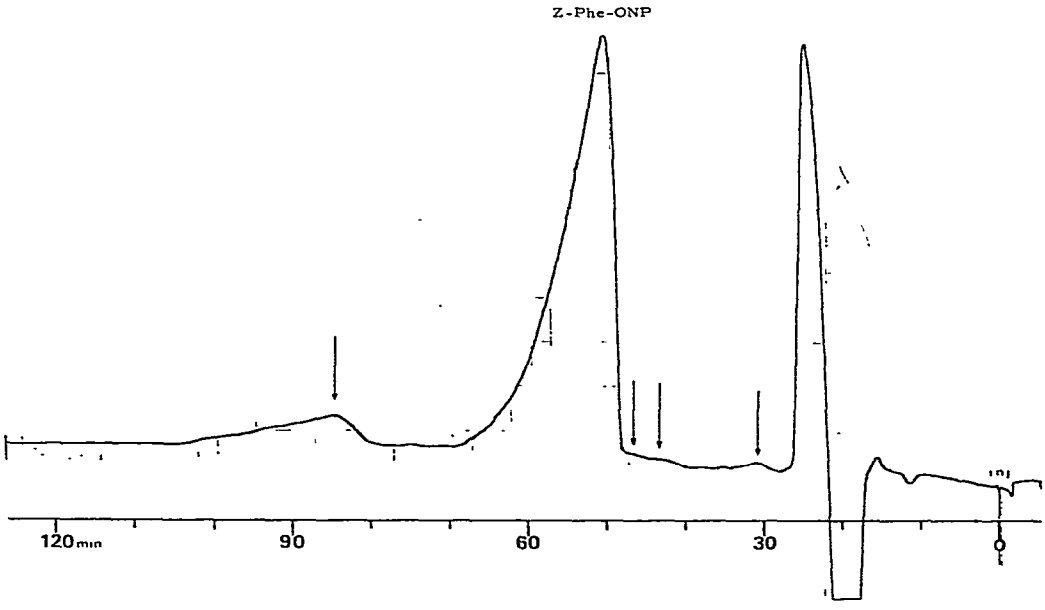


Fig. 6. Chromatogram of Z-Phe-ON*p* effluent from the system, monitored with an RI detector. Flow-rate, 5 ml/min; mobile phase, ethyl acetate–benzene (2:98). The arrows show the side-products and excess reagents.

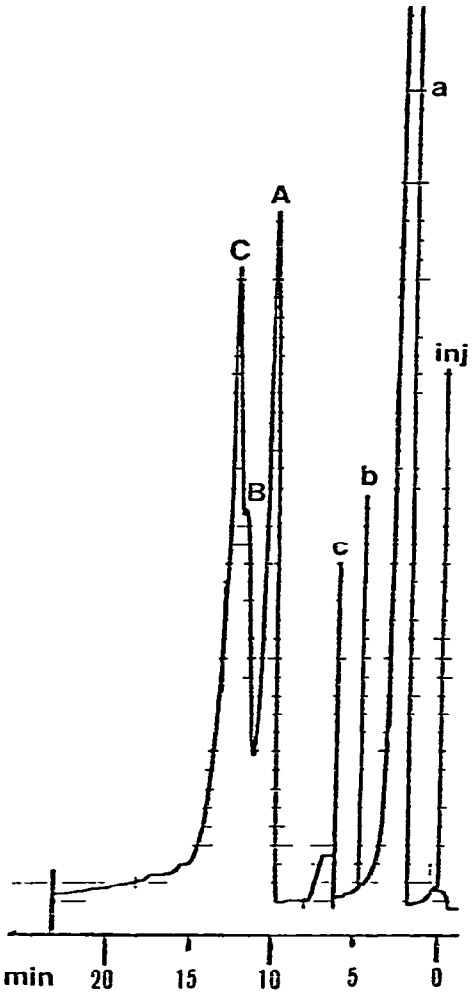


Fig. 7. Extraction and separation chromatogram of a methanolic extract of *Curcuma longa*. Extraction was performed with water-saturated diethyl ether and separation with dry diethyl ether. Flow-rate, 0.8 ml/min. The effluents were monitored with a UV detector set at 254 nm. Peak a shows the ether-soluble materials from the extraction column. The effluent between the injection and line b was concentrated to dryness before line c, at which time the concentrated solute in the evaporator was redissolved in dry diethyl ether and injected into the separation column by an on-line procedure. Peaks A, B, and C show each component included in peak a. Peaks B and C were identified as curcumin and isocurcumin, respectively.

CONCLUSION

The mechanism involving droplet counter-current extraction and liquid–liquid column extraction coupled with chromatographic separation permitted the system to be applied to a wide range of preparative work, converting the previous batch process into the flow procedures. Although there are still some manual operations of valve switching at present, we believe that the programmed flow preparation system will

eventually become completely automated. Its great advantages are as follows: (1) quantitative extraction of the solute from aqueous mixtures; (2) increase in efficiency or number of theoretical plates per unit time; (3) decrease in carry-over loss; (4) continuous running in a closed system by simple operations; and (5) high reproducibility of the results.

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