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Isolation of aristolochic acids HA_sI and HA_sII by preparative liquid chromatography

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ABSTRACT

To isolate the aristolochic acids HA_sI and HA_sII from a crude extract of the *Aristolochia clematitis* L. roots, preparative liquid chromatography was used. A system of reversed phases was applied with a methanol–water–acetic acid mobile phase. Conditions for the extraction of the acids with chloroform from the eluate were also elaborated. The production rate of the chromatographic separation depending on the mobile phase composition was evaluated.

INTRODUCTION

Aristolochic acids, derivatives of 3,4-methylenedioxy-10-nitro-1-carboxyphenanthrene, belong, in addition to chloramphenicol and a few other natural compounds, to substances rarely found in nature that contain a nitro-group. Owing to their biological action [1–5] and their capability of potentiating leukocyte phagocytosis, they are used in the therapy of infectious diseases, although they also have some toxic properties [4,5]. Compounds of this group are difficult to separate, so a mixture of the aristolochic acids is used in therapy.

Aristolochia clematitis L. roots contain six acids of very similar structure [6,7]; HA_sI and HA_sII are prevalent and the remaining four acids are found only in insignificant amounts. They can be isolated by a standard method consisting of methyl esterification followed by separation of the esters on alumina and de-esterification to the free acids. The yield of the de-esterification process is very low, within the range 5–15% [8,9]. Partial degradation occurs, probably owing to the known sensitivity of aromatic nitro compounds to alkalis. Kupchan and Wermser [7] separated a crude mixture of acids and their esters by chromatography using a column packed with silicic acid–Celite (4:1) and a mobile phase of chloroform–ethanol mixtures in various proportions. We failed to reproduce their experiments.

In the method described here, we used high-efficiency preparative reversed-phase liquid chromatography to isolate HA_sI and HA_sII from a mixture of crude acids. A preliminary choice of the mobile phase for the preparative system was made with the use of an analytical system described elsewhere [10].

In the last decade, many studies dealing with optimization of the experimental conditions of preparative elution chromatography have been reported, and general rules were established relating throughput or production rate with the various chromatographic parameters [11–16]. An understanding of these rules diminishes considerably the number of necessary trial-and-error runs, but does not eliminate them completely. The equations given in the literature can be used for the rough estimation of the parameters rather than for their accurate

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calculation. For the latter, an accurate determination of the equilibrium isotherms is necessary. Moreover, the determination of the optimum injection conditions is complicated with samples that are sparingly soluble in the mobile phase [17].

EXPERIMENTAL

Solvents

Methanol, tetrahydrofuran (THF), acetic acid and chloroform of analytical-reagent grade were obtained from POCH (Gliwice, Poland). All solvents were distilled before use. Water was distilled twice.

Apparatus

The apparatus employed for preparative-scale liquid chromatography was equipped with a pump of output up to 270 cm^3/min and a UV detector (254 nm) with a flow cell of 10 mm^3 capacity. A 250 × 13 mm I.D. column was slurry-packed with bonded-phase octadecylsilica, particle size d_p = 10 μm. Approximately 23 g of the material were packed in each column. The surface area of the packing material was 360 m^2/g. Before packing, a final capping with trimethylchlorosilane was performed to minimize the available silanol groups. This material was prepared in the Department of Organic Chemistry, Technical University of Gdańsk.

Sampling the substance was performed by means of a valve with loops of different volumes.

Fraction purity was checked using a Merck–Hitachi liquid chromatograph equipped with a Model L 4250 UV detector, Model L 6200 pump, Model D 2500 integrator and a column (125 × 4.6 mm I.D.) packed with LiChrosorb RP-18, d_p = 5 μm.

Preparation of crude aristolochic acids mixture from plant material

A 1000-g amount of crushed *A. clematitis* L. roots was macerated for 18 h in 10% aqueous formic acid solution. The macerated material was then extracted at room temperature with dichloromethane. The extract thus obtained was filtered and shaken with

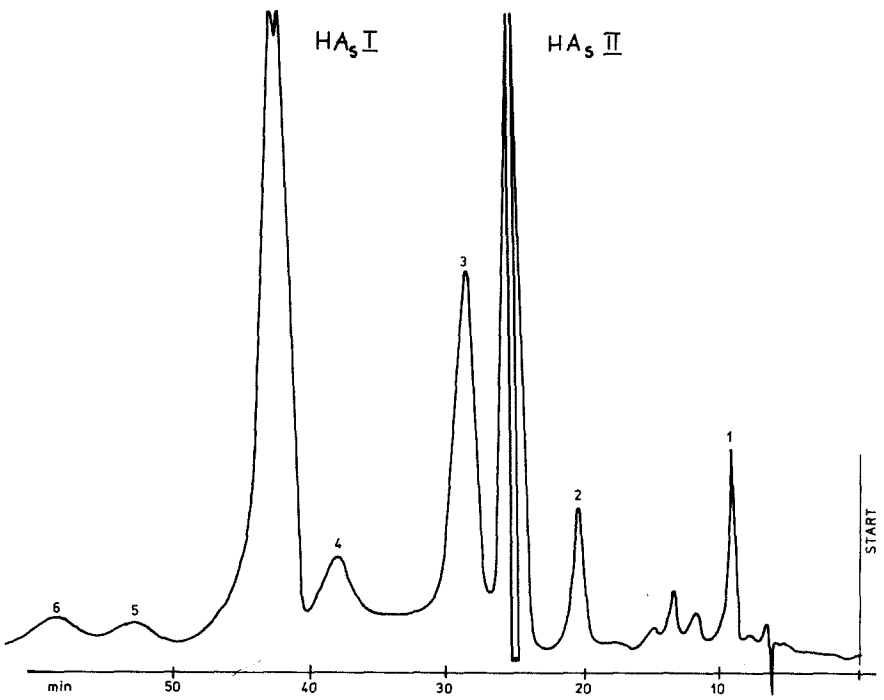

Fig. 1. Analytical chromatogram. Sample, crude mixture of acids; column, 250 × 13 mm I.D., octadecylsilica, d_p = 10 μm; mobile phase, methanol–water–acetic acid (55:45:1, v/v/v); flow-rate, 5.3 cm^3/min; loading, 2 · 10^{-5} mg/g. Detection, UV at 254 nm; sensitivity, 0.08 a.u.f.s.

15% $NaHCO_3$ in the distributer. The aqueous layer was separated and acidified with hydrochloric acid to pH *ca.* 3 and the yellow precipitate was filtered and washed with water until neutral and then dried. The HA_sI and HA_sII content in the crude mixture was up to 24% and 68%, respectively.

Chromatographic procedure

Conditions for preparative separation were chosen on the basis of data obtained for the analytical columns. Comparability of the two systems was obtained by using identical packings in the analytical and preparative separations. The crude mixture of acids dissolves well in THF but hardly at all in methanol and water. A sample was dissolved in THF–methanol (1:1, v/v) with an acid concentration of 20 mg/cm^3 and loaded on to a column. It was confirmed in tests that introducing the sample in drops into the mobile phase did not cause precipitation of components. The column was loaded with amounts of sample from $2 \cdot 10^{-5}$ to 2.8 mg/g. Fractions were manually collected by monitoring the recorder output from the UV detector. They were collected in batches of 10 cm^3 each.

Typical analytical and preparative chromatograms are shown in Figs. 1 and 2. The number of fractions collected depended on the composition of the mobile phase. Substances with retentions higher than that of HA_sI were eluted from the column with methanol (80–120 cm^3). The fractions were successively analysed by the analytical liquid chromatography method using the methanol–water–acetic acid (65:35:1, v/v/v) as the mobile phase. Fractions with concentrations of the isolated components (HA_sI and HA_sII) not lower than 96% were collected, the others being evaporated and rechromatographed. When the column was loaded with a sample amount of 1.3 mg/g, a crystalline lamella-shaped orange precipitate appeared in fractions 11–14 and an amorphous light-yellow precipitate in fractions 20–24 (see Fig. 2).

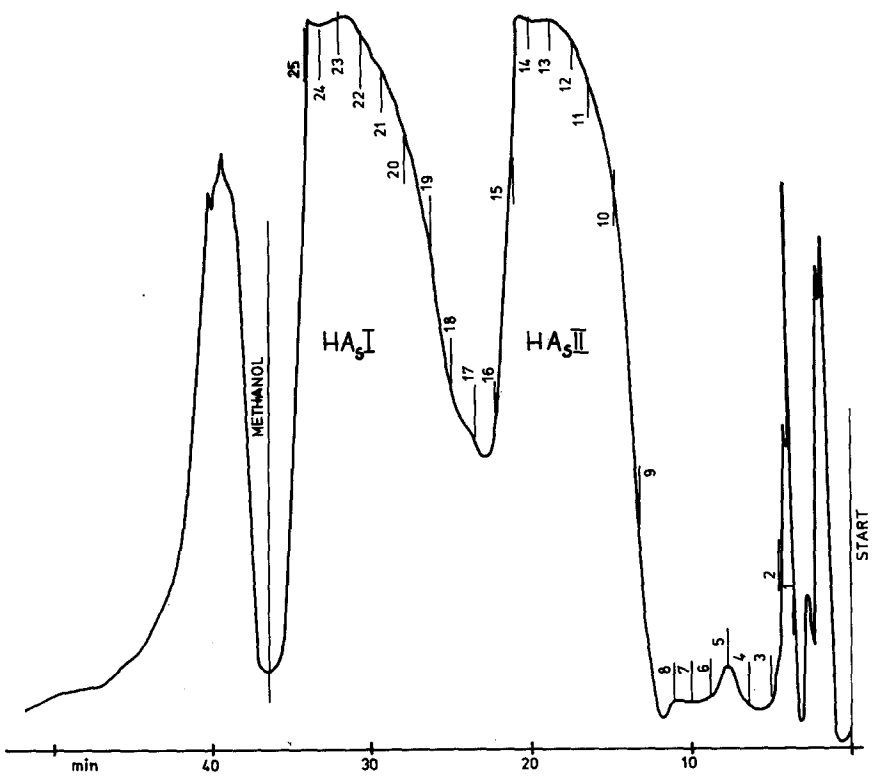

Fig. 2. Preparative chromatogram. Sample, crude mixture of acids; column and mobile phase as in Fig. 1; 25 fractions collected; loading, 1.3 mg/g; flow-rate, 8.3 cm^3/min; sensitivity, 1.28 a.u.f.s.

RESULTS AND DISCUSSION

Selection of mobile phase

The components of the mixture differed considerably with respect to polarity, which renders the selection of an appropriate preparative system difficult, so numerous systems were tested [10]. It has been found that changing the methanol concentration has a substantial effect on retention and selectivity. The values of some retention parameters obtained for various mobile phases are given in Table I. For the most important pairs of substances, *i.e.*, peak 2 and HA_sII, and peak 4 and HA_sI (see Fig. 1), the α factor has the maximum value for mobile phase 2 and one may expect the highest production rate when using this phase.

Selection of sample size

The aim of this work was to obtain multigram amounts of HA_sI and HA_sII. A column of dimensions 250 × 13 mm I.D. (n = 4000) was applied for this purpose. The number of theoretical plates is large enough to ensure preparative resolution using mobile phases 2 and 3. The required number of plates, estimated according to eqn. 5 in ref. 16, for the HA_sI–peak 4 pair was 1200 for mobile phase 2 and about 1800 for mobile phase 3. As the d_p^2/L ratio was fixed, only the amount of sample and the flow-rate of the mobile phase could be chosen. Unfortunately, principles for the choice of the sample size presented elsewhere [11–16] appeared to be unsuitable here owing to the very low solubility of the sample in the mobile phase. Taking into consideration Cox and Snyder's simplifying assumption [16], the maximum sample mass would be 17 mg of HA_sII and 12 mg of HA_sI (mobile phase 3). It was tested experimentally that the solubility of HA_sII in this mobile phase was 0.3 mg/cm^3. The total sample volume should not exceed 17 cm^3, so only a small amount of the solute could be injected. Other mobile phases, containing less methanol, dissolve the acids even worse. Therefore, we injected the sample in a solvent stronger than the mobile phase but in which the sample was readily soluble. The problems resulting from this method of injection are known [18–21], but sometimes the results are reasonable.

Changing the volume of the injected sample solution in THF–methanol within the range 0.6–3.0 cm^3, the optimum sample size for mobile phase 3 was found to be 30 mg. An increase in the volume of the sample at the same concentration above 1.5 cm^3 caused a considerable decrease in resolution and hence the throughput of the acids in the required purity decreased. A sample volume of 1.5 cm^3 was arbitrarily taken as adequate for mobile phases 1 and 2.

Examples of the chromatograms obtained for various column loads are presented in Fig. 3. Table II shows the results achieved with three mobile phases. The aim of these experiments was to check whether a change in the mobile phase strength (in addition to the solubility) does not lead to peak distortion, which could result in deterioration of the resolution.

The production rate was calculated from $P_i = r_i Q_i / t_c A_c$, where $r_i = Q_r/Q_i$ is the recovery, Q_r is the substance mass obtained and Q_i is the substance mass injected in one separation cycle, t_c is duration

TABLE I

CAPACITY FACTORS (k') AND SELECTIVITY COEFFICIENTS (α) FOR SELECTED PAIRS OF SUBSTANCES ACCORDING TO THE ORDER OF THEIR ELUTION OBTAINED FOR MOBILE PHASES CONTAINING VARIOUS AMOUNTS OF METHANOL

Mobile phase: methanol–water–acetic acid (v/v/v)	k'[a]								α[a]	
	1	2	HA_sII	3	4	HA_sI	5	6	HA_sII–2	HA_sI–4
(1) 50:50:1	1.0	5.8	7.5	8.5	13.1	14.1	16.2	18.0	1.29	1.08
(2) 53:47:1	0.8	4.3	6.2	6.5	8.4	10.8	11.4	13.2	1.44	1.28
(3) 55:45:1	0.8	3.1	4.0	4.8	6.5	8.0	9.0	10.6	1.29	1.23

[a] Nos. 1–6 refer to peak numbers in Fig. 1.

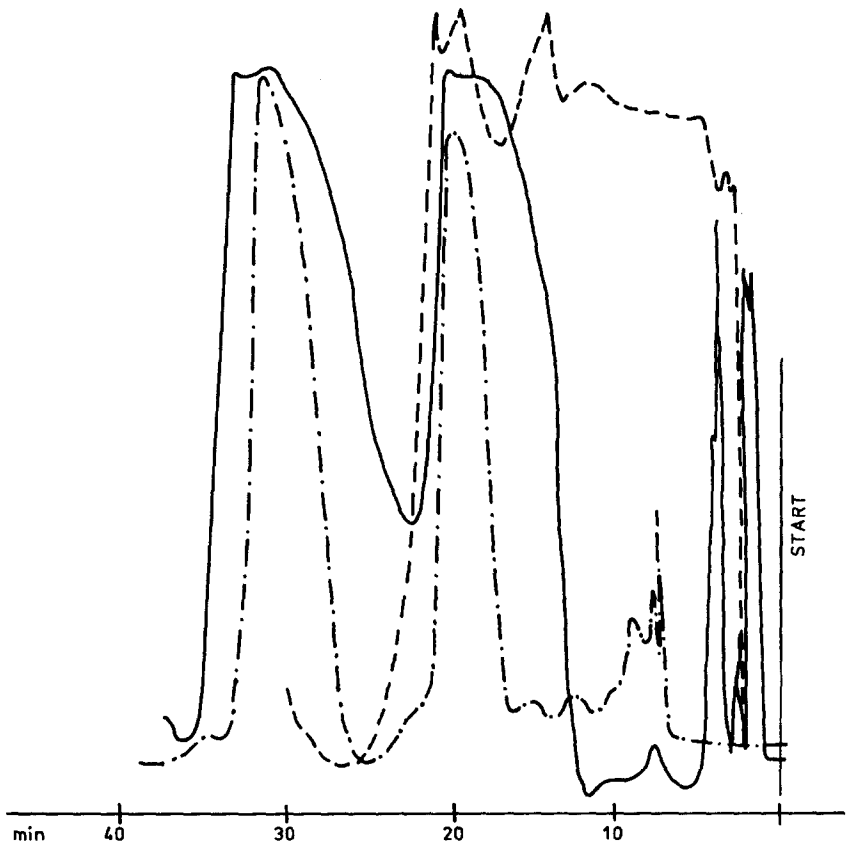

Fig. 3. Preparative chromatogram obtained for various sample volumes: –·–, 0.6 cm^3; ——, 1.5 cm^3; – – –, 3.0 cm^3. Sample concentration, 20 mg/cm^3; sensitivity, 1.28 a.u.f.s. Other conditions as in Fig. 2.

of the separation cycle, $A_c = \pi d_c^2/4$ and d_c is column diameter. The results in Table II lead to the conclusion that the production rate depends, as usual, on α and k', that is, it increases while α increases and k' decreases.

Important parameters are the volume of fractions collected and the total elution volume. When mobile phase 3 was used, the volume of the fraction containing HA_sI decreased by 50% and the total elution volume decreased by a factor of 1.7.

We also examined the mobile phase flow-rate, which influences the process production rate. Fig. 4

TABLE II

PRODUCTION RATES OF ACIDS AT VARIOUS MOBILE PHASE COMPOSITIONS

Mobile phase: methanol–water–acetic acid (v/v/v)	k'_{anal}		Flow-rate (cm^3/min)	Total elution volume (cm^3)	Recovery (%)		Fraction volume (cm^3)		Production rate, P_i ($mg/cm^2 \cdot min$)	
	HA_sII	HA_sI			HA_sII	HA_sI	HA_sII	HA_sI	HA_sII	HA_sI
(1) 50:50:1	7.5	14.1	8.3	448	36	42	30	80	0.10	0.04
(2) 53:47:1	6.2	10.8	8.3	370	45	40	40	60	0.16	0.05
(3) 55:45:1	4.0	8.0	8.3	260	32	40	40	40	0.16	0.07

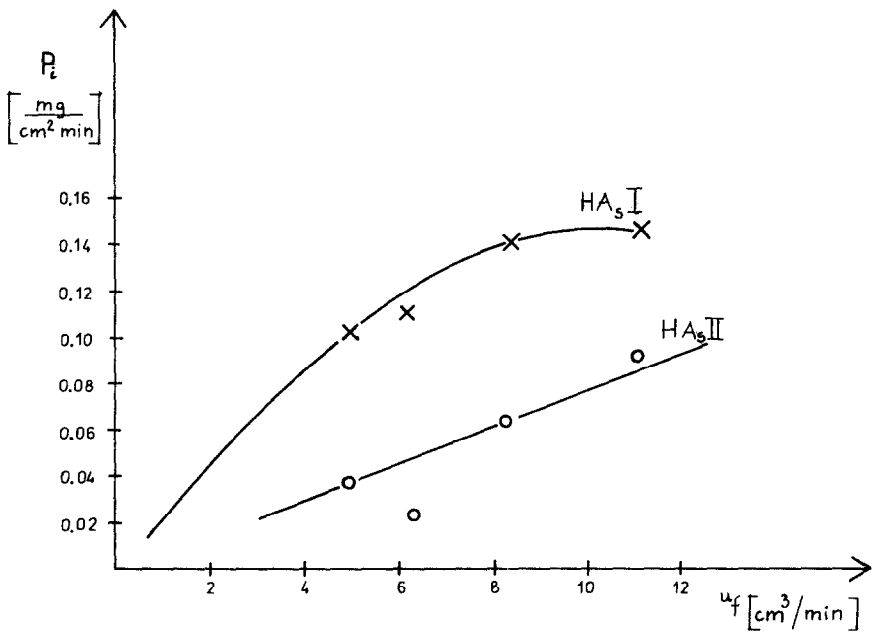

Fig. 4. Dependence of production rate, P_i, on flow-rate. Column and mobile phase as in Fig. 1.

shows the changes in the production rate of HA_sI and HA_sII. The production rate of purified HA_sI is low and it would be profitable to increase the flow-rate. Our conclusions are similar to those in refs. 22 and 23. Unfortunately, technical problems limited the flow-rate of the mobile phase in further experiments to 8.3 cm^3/min. The procedure was repeated ten times with mobile phase 3 and 28 mg of HA_sI and 65 mg of HA_sII were obtained.

As was mentioned before, the solutes began to crystallize from the collected eluates after a few minutes. The crystallization process might be speeded up by cooling, but the crystals formed are very fine and considerable amounts of substances, particularly HA_sII, remain in the filtrate. It is possible to evaporate the solvent using a vacuum evaporator but this takes a long time as the mobile phase contains much water. In addition, the evaporation must be carried out carefully because the acids are surface-active substances and cause excessive foaming. To avoid these difficulties, the acids were extracted from the eluate using various solvents. The best results were achieved with chloroform; 3 × 5 cm^3 were sufficient for complete extraction. This made it possible to diminish the volume of the solvent to be evaporated threefold and the evaporation process was much easier than with methanol–water–acetic acid mobile phase.

In conclusion, it is possible to purify aristolochic acids by liquid chromatography. The low solubility of the sample in the mobile phase might be a substantial difficulty, but this problem was overcome by dissolving the sample in a solvent stronger than the mobile phase. A sample of volume 1.5 cm^3 in a solvent considerably stronger than the mobile phase did not decrease the resolution so much that it would not be possible to obtain acids of the required purity.

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