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Short Communication

Application of high-performance liquid chromatography to the determination of bitter principles of pharmaceutical relevance

O.P. Semenova*, A.R. Timerbaev☆, G.K. Bonn

Department of Analytical Chemistry, Johannes Kepler University, A-4040 Linz, Austria

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Abstract

A rapid HPLC method for the determination of bitter principles of plant origin (BP) was developed. The reversed-phase systems with C_{18} columns and methanol–water mixtures with gradient concentrations used allow the complete separation of seven BP of different types to be achieved in less than 12 min. Several retention models are presented to describe the relationships between the capacity factors of BP and their hydrophobicities and/or the concentration of an organic modifier in the eluent, which are promising for the identification and evaluation of the chromatographic properties of new substances. Predictions of the retention models were found to agree well with the experimental data for different solute, mobile phase and column combinations in both isocratic and gradient elution modes. The method was applied to the determination of verbenalin in drug drops and found to be suitable for routine analysis.

1. Introduction

Bitter principles (BP), *e.g.*, iridoids, seco-iridoids and sesquiterpene lactones, are important natural products showing certain pharmacological properties [1–3]. Because of the growing interest in these substances by the phytopharmaceutical industry in recent years, the quality control of various phytomedicines and the investigation and exact determination of BP in different plant extracts have become important problems.

BP were first routinely separated by thin-layer chromatography [4–7]. However, in many instances the analysis was time consuming and insufficiently effective in detecting substances in small amounts of plant materials. Gas chromatographic analysis of BP is limited owing to their thermal unstability [8] or requires the preliminary derivatization [9]. Therefore, at present, high-performance liquid chromatography (HPLC) is one of the methods of choice for the rapid examination and separation of small amounts of BP. A number of HPLC procedures for determining BP have been reported [10–17], but all these methods are more or less restricted to the analysis of multi-component mixtures of BP, mainly owing to the use of the isocratic elution mode. Further, no attempts have been made to correlate the chromatographic retention of BP with their structure or mobile phase composition.

This paper reports the successful use of reversed-phase HPLC for the separation and de-

* Corresponding author.

☆ On leave from Mendeleev Russian University of Chemical Technology, Moscow, Russian Federation.

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termination of seven BP of different types. The applicability of the method for the rapid analysis of one of BP in a commercially used drug containing plant species is described. In order to establish a means for identifying new bitter substances and to approximate the retention as a function of the eluent composition, retention models based on a BP hydrophobicity parameter were also developed.

2. Experimental

2.1. Apparatus

The Waters (Milford, MA, USA) computer-controlled HPLC system used consisted of two Model 510 HPLC pumps, used as the solvent delivery system, a U6K injector, a Model 484 tunable absorbance detector monitoring at 254 nm and an interface module. A liquid chromatograph consisting of a Waters Model 600E programmable multi-solvent delivery system, a Waters Model 991 computer-controlled photodiode-array detector and a Waters Model 5200 printer–plotter was also used. The eluent flow-rate was 1 ml/min.

2.2. Columns

The columns used were a 250 × 4.6 mm I.D. stainless steel column slurry packed in the laboratory with 5-μm LiChrosorb RP-18 (Merck, Darmstadt, Germany) and an ODS-Hypersil (5 μm) column (250 × 4 mm i.d.) from Österreichisches Forschungszentrum (Seibersdorf, Austria).

2.3. Materials

BP standards were obtained from Sigma (St. Louis, MO, USA) and Carl Roth (Karlsruhe, Germany) and used as methanol solutions. Their structures are shown in Fig. 1. Methanol of analytical-reagent grade (J.T. Baker, Deventer, Netherlands) and doubly distilled water were used as solvents.

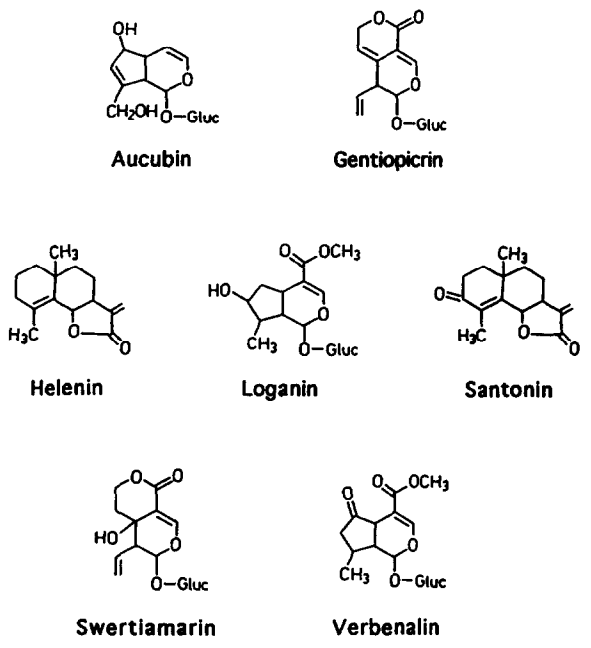

Fig. 1. Structures of the bitter principles examined. Gluc = glucose.

2.4. Procedure

A 20-μl aliquot of Sinupret drops (Bionorica, Neumarkt, Germany), based on a 19% aqueous ethanol solution, was injected directly into the column without any dilution.

3. Results and discussion

3.1. Retention behaviour of bitter principles and its model description

As shown in Fig. 1, the BP studied are related to three classes (iridoids, secoiridoids and sesquiterpene lactones) and differ in glucoside and non-glucoside nature, type of heterocyclic framework and number and position of polar functional groups (hydroxy, oxo, methoxycarbonyl, etc.). Accordingly, they show large differences in retention on reversed-phase columns, which makes their simultaneous separation difficult. For instance, the retention time of BP on the

LiChrosorb column with 50% methanol in the mobile phase varies from 7 to more than 30 min.

To determine the solute retentions and optimize the mobile phase composition in reversed-phase HPLC, calculation methods are widely used [18,19]. These methods can be based on models connecting the retention data with the parameters of solute hydrophobicity and eluent composition. Taking into account that the constituents of the phytochemical preparations under investigation represent different classes of natural substances, it was desirable for the corresponding retention models to be simple and universal.

Retention–structure model

As such a model, we offer here the model based on the simplified criterion of hydrophobicity, H, which was proposed by Shatz and Sakhartova [20] for describing the chromatographic behaviour of pharmaceutical substances with large structural differences:

$$H = n_h - 4n_f^{1/2} \quad (1)$$

where n_h and n_f are the numbers of elementary hydrophobic fragments (*i.e.*, carbon atoms) and polar functional groups of a molecule, respectively. For the BP investigated, the calculated H values are listed in Table 1.

It was found that H is linearly correlated with log k' values according to the equation

$$\log k' = a_0 + a_1 H \quad (2)$$

where k' is the capacity factor, and thereby reflects well the influence of the BP structure on their retention behaviour. For example, the experimental k' values and the k' values calculated from the hydrophobicity parameter are also given in Table 1; the resulting equation obtained by least-squares regression is $\log k' = -(0.269 \pm 0.099) + (0.122 \pm 0.018)H$; $n = 7$; $r = 0.952$; S.D. = 0.113 (r is the regression coefficient and S.D. is the standard deviation). When the retentions (log k') of three or four BP are known at a given eluent composition, Eq. 2 can be used to calculate a_0 and a_1 and approximate the capacity factors for other BP. Reasonably wide applicability of this retention model may be envisaged in the solution of an opposite problem, *viz.*, predicting the structure of an unknown BP from the experimentally obtained solute retention.

Retention–structure and eluent composition model

These combined models should describe the effect of BP structure and mobile phase composition on the capacity factors. The main chromatographic variable for controlling the solute retention is the eluent concentration of an organic modifier, while the simplest characteristic of binary water–organic eluents is the volume concentration of the organic component. Thus,

Table 1
Structural and retention parameters of bitter principals

Compound	Formula	Hydrophobicity parameter	Log k'	
			Exptl.[a]	Calc.[b]
Swertiamarin	$C_{15}H_{20}O_{10}$	2.35	0.055	0.018
Verbenalin	$C_{17}H_{24}O_{10}$	4.35	0.102	0.262
Loganin	$C_{17}H_{26}O_{10}$	4.35	0.138	0.262
Aucubin	$C_{15}H_{22}O_9$	3.00	0.207	0.097
Gentiopicrin	$C_{16}H_{20}O_9$	4.00	0.303	0.219
Santonin	$C_{15}H_{18}O_3$	8.07	0.766	0.716
Helenin	$C_{15}H_{20}O_2$	9.34	0.890	0.870

[a] Column, LiChrosorb RP-18; mobile phase, methanol–water (50:50, v/v).
[b] Log $k' = -(0.269 \pm 0.099) + (0.122 \pm 0.018)H$; $n = 7$; $r = 0.952$; S.D. = 0.113.

combining the parameters H and c (the volume percentage of methanol), we obtained a two-parametric model:

$$\log k' = a'_0 + a'_1 H - a'_2 c - a'_3 Hc \quad (3)$$

which is useful for the *a priori* calculations of organic modifier concentrations providing the desirable mobility of BP. Another important area of such calculations is the determination of initial mobile phase compositions for gradient elution, which could be accomplished with a limited number of isocratic chromatographic experiments (see below).

This model can also be applied to determine the magnitude of the retentions of BP than have not been studied chromatographically. The results of the corresponding calculations are compared with the experimental data in Fig. 2 ($a'_0 = -0.0053$; $a'_1 = 0.170$; $a'_2 = -0.0074$; $a'_3 = -0.0012$; $n = 26$). The error in the calculation of $\log k'$ values averages about 0.09.

Retention–gradient eluent composition model

Mathematical techniques for calculating solute retention parameters in gradient elution chromatography were considered in detail by Jandera and Churacek [19]. For the linear gradient function

$$c = A + Bt \quad (4)$$

where A is the initial concentration of an organic modifier, B is the gradient slope and t is the time after the start of the gradient, and the known coefficients a''_0 and a''_1 of the equation

$$\log k' = a''_0 - a''_1 c \quad (5)$$

the net retention volume V'_R is given by the relationship [21]

$$V'_R = (1/a''_1 B)\log[2.31 a''_1 B V_m \cdot (10^{a''_0} + 10^{a''_1 A})] - (A/B) \quad (6)$$

By using the experimentally obtained $\log k'$ values for 3–5 isocratic eluent compositions, we evaluated the optimal gradient parameters (Eq. 4), determined the coefficients of Eq. 5 and finally calculated by Eq. 6 the retention times of BP. These values are given in Table 2 in comparison with the corresponding experimental data. Hence the described retention model allows one to predict with fairly good accuracy the behaviour of BP in the gradient elution mode from a minimum of isocratic data.

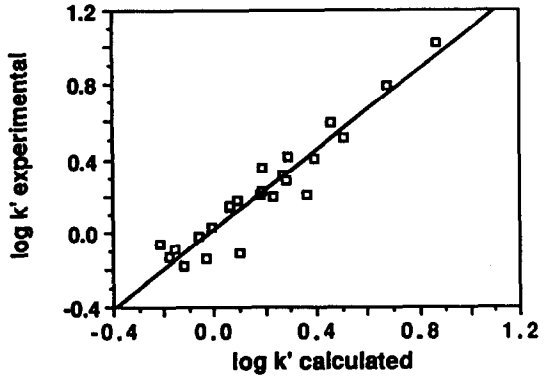

Fig. 2. Prediction of BP retention by Eq. 3. Column, ODS-Hypersil; mobile phase, 10–70% methanol.

3.2. Separation conditions

As mentioned above, it is virtually impossible to separate more than three BP under investigation within a reasonable analysis time using isocratic elution owing to the large differences in the retention. The linear gradients shown in Table 2 resulted in an increase in the number of separands, but the retention times observed were still long. Therefore, to minimize the analysis time and carry-over of long-retained BP, we further optimized gradient elution conditions.

The two main gradient compositions used were the following: (IV) 35% methanol from 0 to 4 min and 95% methanol from 4 to 15 min, and (V) 40% methanol from 0 to 4 min and 95% methanol from 4 to 15 min. Typical chromatograms obtained under the optimized conditions are shown in Fig. 3. Nearly baseline separations for all seven BP (with minimum resolution $R = 1.1$ between verbenalin and loganin for gradient V) can be achieved in less than 12 min.

Table 2
Comparison of experimental and calculated retention times for BP

Compound	t_R (min)					
	Gradient I		Gradient II		Gradient III	
	Exptl.	Calc.	Exptl.	Calc.	Exptl.	Calc.
Aucubin	4.13	4.77	–	–	3.33	4.31
Verbenalin	5.33	4.69	5.66	4.81	4.34	4.16
Loganin	–	–	–	–	6.73	5.08
Gentiopicrin	8.26	8.62	–	–	8.70	7.34
Santonin	14.67	13.71	21.49	18.70	14.44	13.18
Helenin	19.27	19.70	28.68	29.81	21.00	21.80

Column, ODS-Hypersil. Mobile phase, linear variation from 20 to 70% methanol in 20 min (gradient I), from 30 to 60% methanol in 20 min (gradient II) and from 30 to 70% methanol in 30 min (gradient III). $t_0 = 1.65$ min.

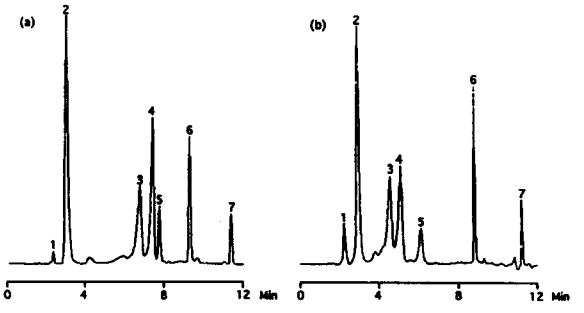

Fig. 3. Typical chromatograms of bitter principles. Column, ODS-Hypersil. Mobile phase: (a) gradient IV; (b) gradient V (for compositions, see text). Peaks (mg/l): 1 = aucubin (12); 2 = swertiamarin (6); 3 = verbenalin (94); 4 = loganin (90); 5 = gentiopicrin (226); 6 = santonin (20); 7 = helenin (136).

3.3. Linearity and detection limits

By plotting the peak heights against the amounts of BP injected, straight lines were obtained under the aforementioned conditions. Table 3 gives the parameters of the calibration graphs with the respective regression coefficients (note the poor correlation for swertiamarin, loganin and gentiopicrin, which might have resulted from insufficient purity of the corresponding standards). The minimum detectable amounts of BP were between 1.2 and 1.5 ng (with a 20-μl injection) at a signal-to-noise ratio of 3. The reproducibility of peak height was checked by six repeated determinations of swer-

Table 3
Parameters of calibration plots for BP

Compound	Interval of calibration (μg injected)	Intercept ×100	Slope $\times 10^3$	Correlation coefficient
Aucubin	1.5–70	10.4	0.07	0.99
Swertiamarin	0.1–0.8	2.4	4.9	0.90
Verbenalin	0.6–3	12.1	18.0	0.97
Loganin	0.5–8	10.0	16.3	0.91
Gentiopicrin	1.9–16	4.7	0.42	0.95
Santonin	0.5–3	0.5	21.8	0.98
Helenin	1.1–6	2.2	4.7	0.97

Chromatographic conditions as in Fig. 3 (gradient IV). $n = 4$–8.

tiamarin and loganin (sample size, 100 ng). The relative standard deviation was 1.4–2.2%.

3.4. Application to drug analysis

In order to evaluate the quantitative performance of the method, commercially used drug drops were analysed. Using a slightly modified gradient programme (25% methanol from 0 to 5 min and 95% methanol from 5 to 15 min), verbenalin was identified in Sinupret drops. This BP is present in *Herba verbenae* [22,23] as one of the main drug components of plant origin used for the preparation of this phytomedicine. Its identity was confirmed by the injection of a drug sample and a drug sample containing an additional small amount of verbenalin, which resulted in an increase in the height of the respective peak (Fig. 4). Additional confirmation was obtained from a UV spectrum of the corresponding peak recorded on-line with a photodiode-array detector and by capillary zone electrophoresis (the results will be presented in future papers).

The calibration graph for verbenalin of detector response (y) versus concentration (x, mg/l) was linear ($y = -0.0201 + 0.00012x$; $r^2 = 0.996$) in the concentration range 0.08–0.6 mg/l. The detection limit was found to be 51 μg/l. On the basis of triplicate analyses, the content of verbenalin in the drug was determined to be 148 ± 17 mg/l. From the results presented in Table 4, it can be seen that this technique produces satisfactory recovery results for the purpose of the determination of BP in phytotherapeutic products.

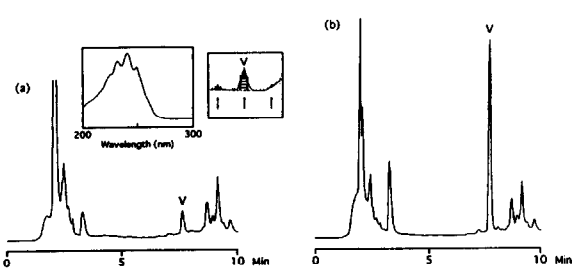

Fig. 4. Chromatograms of (a) a drug sample and (b) the drug spiked with 436 μg/ml of verbenalin (V). Column, ODS-Hypersil. Mobile phase as in text. Insets show the UV spectrum and peak purity recorded by the photodiode-array detector.

Table 4
Recovery of verbenalin added to a drug sample

Amount added (mg/l)	Amount found (mg/l)	Recovery (%)
155	150	96.8
390	412	105.6
436	429	98.4

5. Conclusions

HPLC allows the rapid determination of BP in phytotherapeutic products. Coupled with a photodiode-array detector, this method seems to be fairly promising for use in sophisticated phytopharmaceutical industry analyses. Investigations are in progress on the same application of high-performance capillary electrophoresis as a more efficient, economic and less sample-consuming alternative to HPLC.

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