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TLC and HPLC characteristics of desacetylmatricarin, leucodin, achillin and their 8α-angeloxy-derivatives

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Received December 23, 2002, accepted January 26, 2003

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Pharmazie 58: 487–490 (2003)

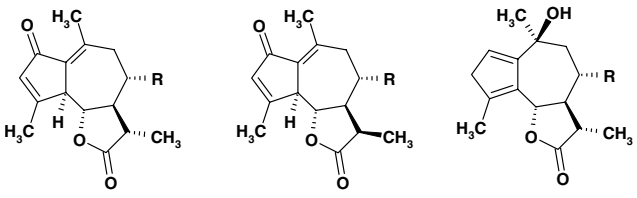
Five guaianolides, including two pairs of isomers, from a Hungarian taxon of the *Achillea millefolium* group were characterized analytically. Different chromatographic systems on TLC and HPLC were developed for the analysis of these compounds. TLC of leucodin, 8α-angeloxy-leucodin, achillin, 8α-angeloxy-achillin and desacetylmatricarin was performed on silica gel using dichloromethane-acetone and cyclohexane-ethylacetate mixtures as mobile phases. HPLC on stationary phases as LiChrospher®RP2, LiChrospher®RP8, LiChrospher®RP18e, Hypersil BDS C_{18} and Aquasil C_{18} required isocratic and gradient systems with different methanol-water mixtures as mobile phases. The presented R_F values and retention times allow the identification of the respective 2-oxo-guaianolides which are marker substances for certain non-proazulene containing species. Their TLC and HPLC fingerprints are compared to those of proazulene containing species and are relevant for quality control.

1. Introduction

2-Oxo-guaianolides are naturally occurring compounds with various biological activities which can be extracted from different herbal remedies prepared from the respective drugs. Desacetylmatricarin (**3**), leucodin (**1**) and achillin (**4**) are described frequently for Asteraceae as for example in *Achillea*, *Artemisia*, *Tanacetum* or *Taraxacum*. **1**, **3** and **4** were shown to possess anti-allergic activity by evaluation of their inhibitory effects on the β-hexosaminidase release from cells of a rat mast cell line [1]. Hydroxylated compounds of this type have also been mentioned with respect to effects on the arachidonic acid metabolism in cellular systems [2] and with respect to the use as natural herbicides [3]. Recently two new derivatives, 8α-angeloxy-leucodin (**2**) and 8α-angeloxy-achillin (**5**), have been isolated from a Hungarian taxon of the *Achillea millefolium* group [4]. It was the first time that all five compounds were isolated from one plant, informations about analytical data in literature are poor up to now. To separate the respective compounds which show only slight structural differences TLC and HPLC systems were developed in which R_F values and retention times are well distinguishable. Up to now **1**, **2**, **4** and **5** which have been detected in *Achillea* samples from Hungary and Romania never occurred together with proazulenes which are required by the European Pharmacopoea. They are stable and do not contribute to the blue colour of the essential oil. Their presence besides the proazulenes **6**–**8** (artabsin-derivatives) in a "*Herba*" drug or in plant extracts indicates that mixtures of proazulene and non-proazulene containing taxa were used. The presented analytical data allow the quick and reliable identification of the guaianolides **1**–**5** in plant extracts and contribute to the quality control of the drug "*Herba Millefolii*". TLC and HPLC comparisons of *Achillea collina* (proazulene containing) and the Hungarian taxon (non-proazulene containing) are shown.

2. Investigations, results and discussion

As a general characteristic the compounds **1**–**5** are easily to detect by fluorescence quenching under $UV_{255\,nm}$ due to their λ_{max} at 260 nm and do not give any colouring with spraying reagents as acetic acid-phosphoric acid reagent (see Fig. 1b) or sulphuric acid-anisaldehyde reagent. In the TLC system dichloromethane-acetone (9 : 1) which is generally used for fingerprint analyses of *Achillea* species [6] the five compounds **1**–**5** revealed three spots under $UV_{255\,nm}$. Compound **3** showed high polarity with a R_F value of 0.30 due to its free hydroxyl group. Substitution of the latter with angelic acid led to a significant decrease



1 (R=H)
2 (R=Oang)
3 (R=OH)

4 (R=H)
5 (R=Oang)

6 (R=Oac)
7 (R=Otigl)
8 (R=Oang)

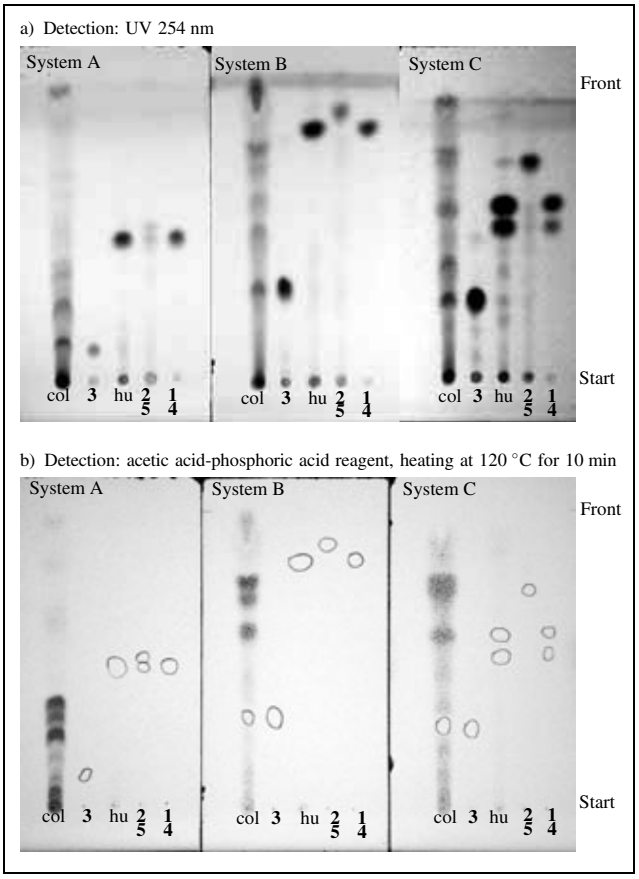


Fig. 1: TLC of *Achillea collina* (col), compound **3**, a Hungarian sample of the *Achillea millefolium* group (hu), mixture of the compounds **2/5** and mixture of the compounds **1/4**. Stationary phase: silica gel 60 Merck (0.25 mm); mobile phases (see experimental): dichloromethane-acetone (95 + 5) – system A; dichloromethane-acetone (9 + 1) – system B; cyclohexane-EtOAc (1 + 1) – system C; detection: a) UV 254 nm b) acetic acid-phosphoric acid reagent (flourescence quenching marked by pencil)

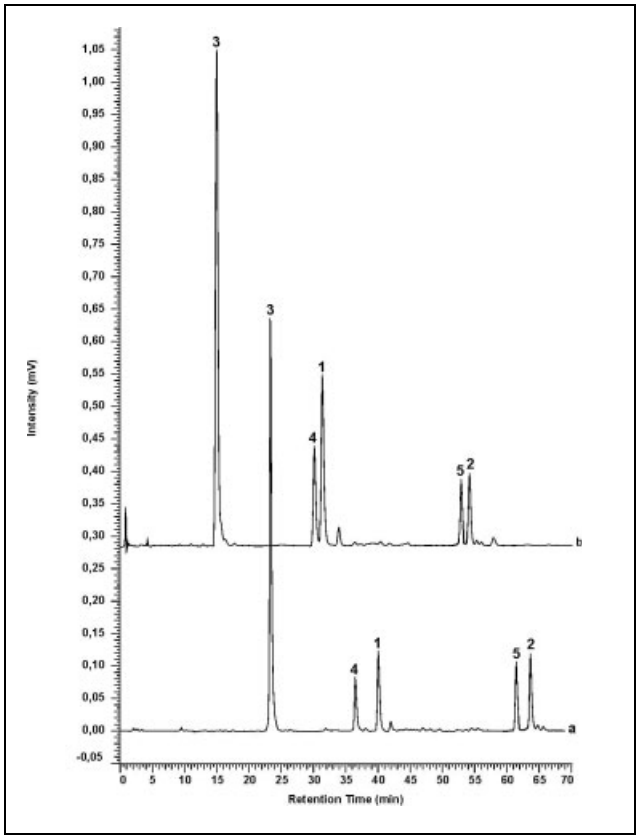


Fig. 2: HPLC chromatogram of the pure compounds **1**–**5**. a) Stationary phase: Hypersil BDS C_{18} 5 µm 250 × 4 mm; mobile phase: methanol-water gradient system, start at 20% (v/v) up to 80% in 90 min, rate: 0.66%/min; detection: 260 nm; b) Stationary phase: Zorbax SB C_8 3.5 µm 75 × 4.6 mm; mobile phase: methanol-water gradient system, start at 20% (v/v) up to 80% in 90 min, rate: 0.66%/min; detection: 260 nm

of polarity with a R_F value of 0.89 for **2** and **5**. **1** and **4** lack the hydroxyl group and reveal a R_F value of 0.83. The separation of the both pairs of stereomers which showed different orientation of their methyl group in position 12 (CH_3-13 α-orientated in **1** and **2**: ''leucodins'', CH_3-13 β-orientated in **4** and **5**: ''achillins'') could not be achieved in this system, but the mobile phase cyclohexane-ethylacetate (1 : 1) resulted in $R_F = 0.51$ for achillin (**4**) and $R_F = 0.59$ for leucodin (**1**). A separation of the respective 8α-angeloxy derivatives **2** and **5** in this system failed, whereas dichloromethane-acetone (95 : 5) showed a R_F of 0.45 for 8α-angeloxy-achillin and 0.49 for 8α-angeloxy-leucodin (see Fig. 1).

For HPLC analysis RP2, RP8 and RP18 materials with different qualities (LiChrospher, Zorbax, Hypersil and Aquasil) were tested as stationary phases. The polar desacetylmatricarin (**3**) eluted first followed by achillin (**4**), leucodin (**1**), 8α-angeloxy-achillin (**5**) and 8α-angeloxy-leucodin (**2**). The interaction of **1**–**5** with RP2 seemed to be less compared to RP8 or RP18 material resulting in shorter retention times and overlapping peaks. On Aquasil® which offers polar as well as apolar groups to the analyts **1**–**5** showed tailing and were therefore not baseline separated. The demand to separate all five compounds with their different polarities in one HPLC run required a gradient system. By starting at 20% methanol and increasing the methanol ratio with a rate of 0.66% per minute reasonable retention times and good separations were achieved. Especially the phases Zorbax C_8 and Hypersil C_{18} turned out to be best suitable for the stereomers leucodin (**1**) and achillin (**4**) respectively 8α-angeloxy-leucodin (**2**) and 8α-angeloxy-achillin (**5**) which appear as sharp peaks without tailing (Fig. 2). Their chromatographic behaviour on RP corresponds to that on silica gel: the ''achillins'' **4** and **5** come earlier, respectively show smaller R_F values than the leucodines **1** and **2**. This leads to the conclusion that β-orientation of the methyl group in position 13 slightly increases polarity.

The HPLC (Fig. 3) and TLC (Fig. 1) fingerprints show clear differences between the proazulene containing *Achillea collina* and the proazulene free Hungarian sample. Only the proazulenes give an intensive blue colour with acetic acid-phosphoric acid reagent on TLC. Desacetylmatricarin (**3**) is mostly present in proazulene containing species, whereas the compounds **1**, **2**, **4** and **5** seem to be marker substances for certain proazulene free taxa. The different UV maxima of the proazulenes ($\lambda_{max} = 220$ nm) and of the 2-oxo-guaianolides ($\lambda_{max} = 260$ nm) require a parallel detection at both wavelengths during HPLC analysis.

The presented TLC and HPLC methods allow a qualitative check on the 2-oxo-guaianolides leucodin (**1**), 8α-angeloxy-leucodin (**2**), desacetylmatricarin (**3**), achillin (**4**) and 8α-angeloxy-achillin (**5**). The developed methods provide a good separation of the stereomers achillin/leucodin and their 8α-angeloxy-derivatives, respectively. With help of the presented data a quick separation and identification of these compounds in plant extracts or enriched fractions

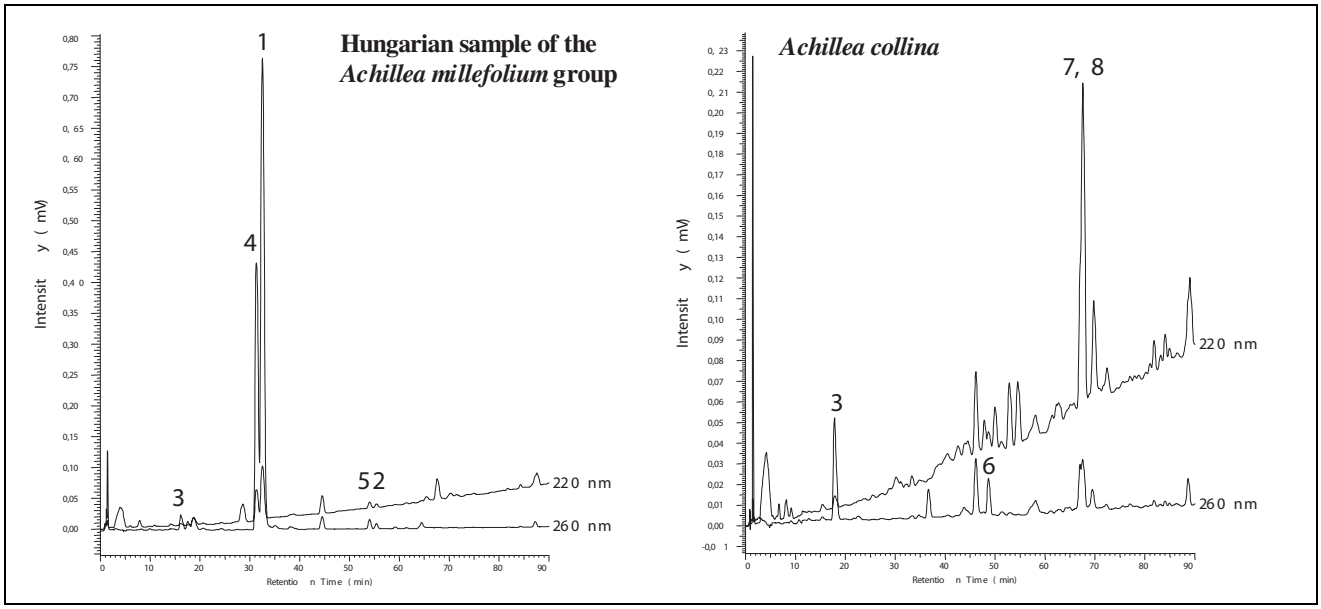


Fig. 3: HPLC chromatograms of *Achillea collina* and a Hungarian sample of the *Achillea millefolium* group. Stationary phase: Zorbax SB C_8 3.5 µm 75 × 4.6 mm; mobile phase: methanol-water gradient system, start at 20% (v/v) up to 80% in 90 min, rate: 0.66%/min; detection: 220 nm and 260 nm

is possible. Whereas in proazulene containing species desacetylmatricarin (**3**) is mostly present they do not contain the compounds **1**, **2**, **4** or **5**. The detection of these substances besides proazulenes in a plant extract hints that species of different quality were used. This is of relevance with respect to the quality control of the drug "*Herba Millefolii*" which should contain at least 0.02% proazulenes according to the European Pharmacopoea.

Table 1: Sum formulas, molecular weights, λ_{max} and R_F of 1–5

Compd.		Sum formula	Molecular weight	λ_{max} (nm)	R_F CH_2Cl_2-acetone (95 : 5, v/v)	R_F CH_2Cl_2-acetone (9 : 1, v/v)	R_F cyclohexane-EtOAc (1 + 1, v/v)
1	Leucodin, desacetoxymatricarin, leucomisin	$C_{15}H_{18}O_3$	246	260	0.46	0.83	0.59
2	8α-Angeloxy-leucodin	$C_{20}H_{24}O_5$	344	260	0.49	0.89	0.73
3	Desacetylmatricarin, austricin, austrisin	$C_{15}H_{18}O_4$	262	260	0.10	0.30	0.26
4	Achillin	$C_{15}H_{18}O_3$	246	260	0.46	0.83	0.51
5	8α-Angeloxy-achillin	$C_{20}H_{24}O_5$	344	260	0.45	0.89	0.73

Table 2: Retention times t_R (min) and retention factors k[a] of 1–5 in different HPLC systems

Stationary phase	Mobile phase	**3**		**4**		**1**		**5**		**2**	
		t_R	k	t_R	k	t_R	k	t_R	k	t_R	k
LiChrospher 100 RP8	30% isocratic	14	4.8	65	26.1	67	26.9	–	–	–	–
5 µm; t_0 = 2.4 min[b]	50% isocratic	4	0.7	8	2.3	8	2.3	37	14.4	38	14.8
	0.33%/min	36	14.0	60	24.0	61	24.4	–	–	–	–
	0.66%/min	27	10.3	41	16.1	42	16.5	63	25.3	64	25.7
Zorbax SB-C_8 3.5 µm; t_0 = 0.9 min[b]	0.66%/min	15	15.7	30	32.3	31	33.4	53	57.9	54	59.0
LiChrospher 100 RP18 5 µm; t_0 = 2.1 min[b]	0.66%/min	22	9.5	38	17.1	40	18.0	60	27.6	61	28.0
LiChrospher 100 RP18e 5 µm; t_0 = 2.7 min[b]	0.66%/min	23	7.5	37	12.7	40	13.8	61	21.6	63	22.3
Hypersil BDS C_{18} 5 µm; t_0 = 2.6 min[b]	0.66%/min	23	7.8	37	13.2	40	14.4	62	22.8	64	23.6
LiChrospher RP2 7 µm; t_0 = 2.6 min[b]	0.66%/min	9	2.5	17	5.5	18	5.9	36	12.8	38	13.6
Aquasil C_{18} 5 µm; t_0 = 2.8 min[b]	0.66%/min	31	10.1	48	16.1	49	16.5	67	22.9	68	23.3

[a] Calculated: $k = \frac{t_R - t_0}{t_0}$

[b] For the determination of t_0 thiourea was used

3. Experimental

3.1. Chemicals

Desacetylmatricarin, leucodin, achillin, 8α-angeloxy-leucodin and 8α-angeloxy-achillin were isolated from *Achillea millefolium* s.l., collected in Szekszárd, South Hungary, in July 1997 [4]. Methanol (HPLC grade) was obtained from Merck (Darmstadt, Germany). All other chemicals were analytical reagent grade.

3.2. Plant material and sample preparation

For fingerprint comparisons *Achillea collina* as proazulene containing representative, collected in 1995 at Vösendorf, Lower Austria, and single plants from a Hungarian taxon of the *Achillea millefolium* group as non-proazulene containing representative, collected in 1997 at Szekszárd, South Hungary, were used. Specimens of both origins are deposited at the Herbarium of the Institute of Pharmacognosy, University of Vienna (Austria). For the single plant analyses 100 mg air dried flower heads were extracted for 10 min with 1 ml CH_2Cl_2 by ultrasound at room temperature. The solution was filtered via a pipette provided with a small ball of cotton wool at the tip, if necessary dried with Na_2SO_4 and evaporated under nitrogen. The residue was redissolved in 500 µl CH_2Cl_2, 10–20 µl thereof were analyzed by HPLC and TLC. As the proazulenes easily degrade under the influence of light, oxygen and temperature the solutions should always be prepared freshly. In contrast, the 2-oxo-guaianolides are characterized by high stability, these plant extracts can be stored after removement of dichloromethane at +4 °C.
The pure substances **1**–**5** were dissolved in methanol and analyzed by TLC and HPLC.

3.3. HPLC

HPLC was performed on a Merck Hitachi system consisting of a Rheodyne injection unit, a LaChrom pump L-7100, a LaChrom diode array detector L-7450 (monitoring wavelengths 220 nm and 260 nm, respectively) and an interface D-7000 (Merck, Vienna, Austria). UV spectra were recorded on line in methanol-water by DAD detection during the HPLC runs. All computations were performed using the Merck D-7000 HSM data system. Separations were carried out on following stationary phases: 250 × 4 mm Hewlett-Packard LiChrospher 100 (Merck)-RP8 5 µm, 75 × 4.6 mm Hewlett-Packard Zorbax (DuPont) SB-C_8 3.5 µm, 250 × 4 mm Hewlett-Packard LiChrospher 100 (Merck)-RP18 5 µm, 250 × 4 mm Hewlett-Packard LiChrospher 100 (Merck)-RP18e 5 µm, 250 × 4 mm Hypersil BDS-C_{18} 5 µm, 250 × 4 mm LiChrospher RP2 7 µm, 250 × 4.6 mm Keystone Scientific Aquasil C_{18} 5 µm. The mobile phases consisted of varying methanol-water mixtures (v/v), flow rate was 1 ml/min at room temperature. System 1: 30% methanol (isocratic system); system 2: 50% methanol (isocratic system); system 3: start at 20% methanol to 80% methanol in 180 min (linear gradient; rate = 0.33%/min); system 4: start at 20% methanol to 80% methanol in 90 min (linear gradient; rate = 0.66%/min). The retention times of the compounds in the respective systems are summarized in Table 1.

3.4. TLC

TLC Silica gel plates (Merck, Germany, 0.25 mm), were developed with different mobile phases at room temperature. System A: CH_2Cl_2-acetone (95 : 5, v/v). System B: CH_2Cl_2-acetone (9 : 1, v/v). System C: cyclohexane-EtOAc (1 : 1, v/v). After development at room temperature chromatograms were examined under $UV_{255\,nm}$ and subsequently sprayed with acetic acid-phosphoric acid reagent (0.25 g dimethylamino-benzaldehyde, 50 g acetic acid, 5 g phosporic acid 85%, 20 ml water [5]).

Acknowledgements: Dr. Mucaji was financially supported by a grant of the University of Vienna during his stay in Vienna.

References

1 Ho, Ch.; Choi, E. J.; Yoo, G. S.; Kim, K.-M.; Ryu, S. Y.: Planta Med. **64**, 577 (1998)
2 Silván, A. M.; Abad, M. J.; Bermejo, P.; Villar, A.: Planta Med. **64**, 200 (1998)
3 Macías, F. A.; Galindo, J. C. G.; Castellano, D.; Velasco, R. F.: J. Agric. Food Chem. **48**, 5288 (2000)
4 Glasl, S.; Mucaji, P.; Werner, I.; Presser, A.; Jurenitsch, J.: Z. Naturforsch. **57c**, 976 (2002)
5 Stahl, E.: Dünnschichtchromatographie, Reagens Nr. 65, p. 825, Springer Verlag, Berlin-Heidelberg-New York 1967
6 Glasl, S.; Kastner, U.; Jurenitsch, J.; Kubelka, W.: J. Chromatogr. B **729**, 361 (1999)