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Quantitative determination of cucurbitacin E and cucurbitacin I in homoeopathic mother tincture of *Gratiola officinalis* L. by HPLC

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"Hedgehyssop" *Gratiola officinalis* L. (Scrophulariaceae) is found as an ingredient in homeopathic remedies. Among the active compounds found in *G. officinalis*, the cucurbitacines constitute a group of triterpenoid substances which are well-known for their bitterness and toxicity. Due to the toxicity of the cucurbitacin's aglycones it becomes necessary to determine the content of the aglycones and glycosides that may be responsible for the pharmacological activity and toxicity in homeopathic tinctures according to the European Pharmacopeia guidelines. In this context a HPLC method was developed for the identification and determination of cucurbitacin E and I in homeopathic mother tinctures. To evaluate the total concentration of the aglycones, cucurbitacin E and I formed after hydrolysis we determined the concentration of both compounds after enzymatic hydrolysis with β-glucosidase *in vitro*. Reversed-phase HPLC with a Eurospher C_{18} column with precolumn and acetonitrile-water gradient system as the mobile phase proved to be suitable for direct determination of both aglycons, cucurbitacin E and I in Gratiola-mother tinctures. The contents of cucurbitacin E, cucurbitacin I, cucurbitacin E glycoside and cucurbitacin I glycoside were found as 0.0065%, 0.0031%, 0.0011% and 0.0006%, respectively in Gratiola-mother tincture prepared according to method 2a HAB.

1. Introduction

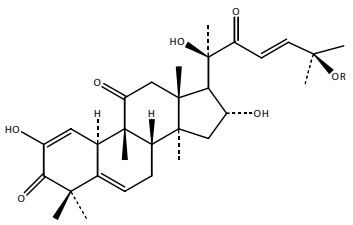
Gratiola officinalis L. is a plant which belongs to the family of Scrophulariaceae. It is the origin of a homeopathic mother tincture monographed in HAB 2007 (German Homeopathic Pharmacopea/Homöopathisches Arzneibuch 2007). Besides, the dried aboveground parts (Herba Gratiolae) are found as ingredient in homeopathic remedies (Wiesenauer 1996).

A number of different cucurbitacin derivatives, such as elateranide (glucoside of cucurbitacin E), desacetyl-elaterinide (glucoside of cucurbitacin I), and the aglycones cucurbitacin E and cucurbitacin I have been isolated from the plant *Gratiola officinalis* L. (Gmelin 1967; Bauer and Wagner 1983; Sturm and Stuppner 2000). The pharmacological activities of cucurbitacin-containing plants have been known since ancient times. Cucurbitacines are particularly known in folk medicine for their strong purgative, antiinflammatory, and hepatoprotective activities (Lavie and Glotter 1971). Moreover, the natural cucurbitacines constitute a group of triterpenoid substances which are well-known for their bitterness and toxicity (Chen et al. 2005). It has therefore been important to determine the content of free cucurbitacins in herbal remedies. Among the β-glycosidases found in the plant, one of the most active enzymes is "elaterase". This enzyme is, depending on the conditions used for the production of homeopathic mother tinctures and other extracts able to hydrolyse the glycosides and therefore lead to the increase in the amount of aglycons found in plants. Glycosidases are considered as very stable enzymes also under different extraction conditions (Kreis 2007). The cucurbitacines, particularly cucurbitacin E, have received great attention because of its cytotoxic and anti-proliferative effects (Duncan et al. 1996; Fuller et al. 1994). Moreover cucurbitacin glycosides are supposed to be responsible for the cardiotoxic activity of "Herba Gratiolae" (Müller and Wichtl 1979).

Because of the toxicity of cucurbitacines aglycones it becomes necessary to determine the content of the aglycons and glycosides in homeopathic tinctures according to the European Pharmacopeia guidelines.

2. Investigations, results and discussion

The method used in this study allowed the determination of cucurbitacin E and I in Gratiola-mother tinctures. Reversed-phase HPLC with a Eurospher C_{18} column with precolumn and acetonitrile-water gradient system as the mobile phase proved to be suitable for direct determination of cucurbitacin E and cucurbitacin I. The peaks were identified by comparison of the retention times and UV spectra with that of the standards (Figs. 1–3).



R=H Cucurbitacin I
R=Ac Cucurbitacin E

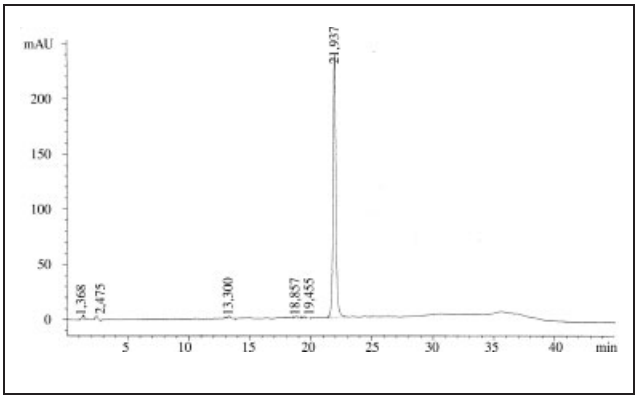


Fig. 1: HPLC-chromatogram of cucurbitacin I

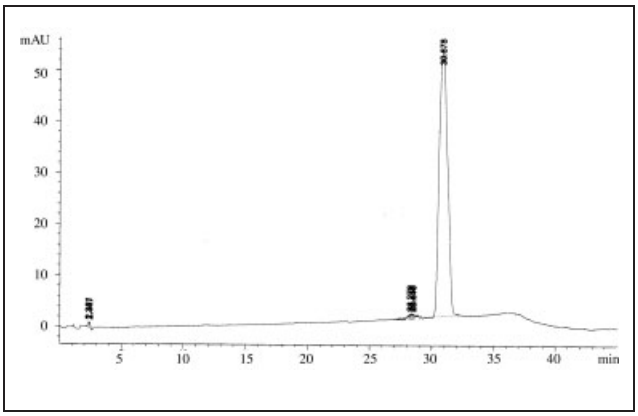


Fig. 2: HPLC-chromatogram of cucurbitacin E

To evaluate the total concentration of the aglycones cucurbitacin E and I formed after complete hydrolysis in the mother tinctures we determined the concentration of both compounds after enzymatic hydrolysis with β-glucosidase *in vitro*. Preliminary studies have been carried out to establish the most suitable conditions for enzymatic hydrolysis. In this context, assays have been performed at 37 °C to find out the optimal period, the amount of enzyme necessary for the hydrolysis and whether shaking would be necessary or not for the reaction to take place. At 37 °C and without shaking of the reaction mixture, 24 h for the incubation time and 150 U enzyme/ml tincture were determined as the optimal conditions for the hydrolysis of cucurbitacin glycosides present in the mother tincture.

The results are shown in the Table. The difference between the aglycone content before and after the treatment of the mother tincture with β-glucosidase allows to calculate the content of cucurbitacin E-glucoside (11.33 μg/ml) and cucurbitacin I-glucoside (5.7 μg/ml). The low amount of glycosides found in the mother tincture might be explained by the presence of the β-glucosidase elaterase in the plant which cleaves the glycosides during the manufactoring procedures for preparation of the mother tincture. Elaterase, is a very active β-glucosidase and was identified in *Gratiola officinalis* L. It is known to hydrolyse glycosides rapidly to free aglycons (Gmelin 1967), but in the homeopathic tincture the activity seems to be stopped. Our results indicate that not all glycosides are cleaved during the preparation of the mother tincture, approximately 15% of cucurbitacine glycosides remain in the alcoholic tincture and can be cleaved by addition of β-glucosidase.

Previously, in a study by Gmelin (1967) cucurbitacin E (0.08 %) and cucurbitacin I (0.02 %) have been isolated from the methanol extract of fresh *Gratiola officinalis* L. Moreover, in a study carried out with *Gratiola*-mother tincture (§2 HAB 34), the cucurbitacin glycosides have not been detected and the aglycones have been found in trace amounts only (Bauer and Wagner 1983). In our study, we have found that the content of cucurbitacin E was higher than that of cucurbitacin I (Table). The differences between the results obtained in the present study and previous investigations might be due to the different preparation of the mother tincture. The longer the time between harvesting and production (addition of alcohol for the preparation of the tincture) the lower the content of cucurbitacin glycosides.

A further deacetylation of cucurbitacin E (and/or its glycoside) to cucurbitacin I (and/or its glycoside) was not observed during the β-glucosidase treatment. Only under drastic conditions (1N HCl and incubation at 100 °C for 60 min) an increase in cucurbitacin I concentration could be observed (results not shown).

Summarizing the results we found in the *Gratiola*-mother tincture a content of 0.0065% cucurbitacin E and 0.0031% cucurbitacin. The corresponding contents of cucurbitacin E glycoside was 0.0011 and of cucurbitacin I glycoside 0.0006%. Taken all together, the total amount of cucurbitacin E and I derivatives is in the range of approximately 0.011% (app. 0.1 mg/ml). Laxative activity is induced by the cucurbitacin E glycosides in doses of 2 mg/kg body weight and cu-

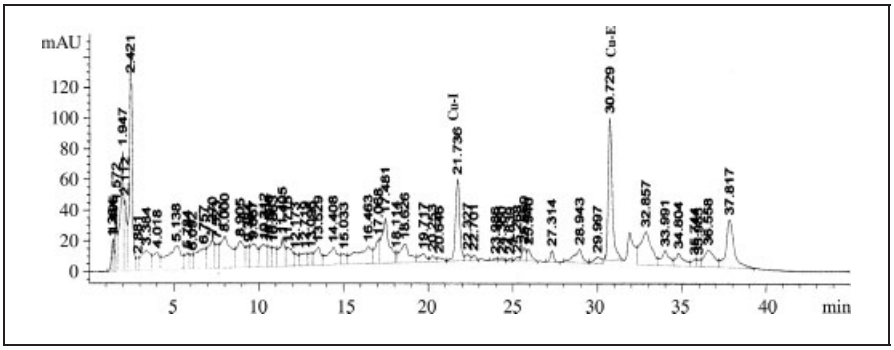


Fig. 3:
HPLC-chromatogram of *Gratiola officinalis* mother ticture (Cu-I: cucurbitacin I, Cu-E: cucurbitacin E)

Table: Contents of cucurbitacin E and cucurbitacin I in mother tincture before and after the treatment with β-glucosidase

Gratiola officinalis V2A URT	Cucurbitacin E content (μg/ml) ± SD	Cucurbitacin I content (μg/ml) ± SD
Mother tincture	64.80 ± 1.20	30.63 ± 1.43
β-glucosidase treated mother tincture	76.13 ± 1.62	36.33 ± 1.43

The results are expressed as the mean of triplicate determinations
SD: standard deviation of the mean

curbitacin I glycoside at 25 mg/kg body weight (Le Men et al. 1969). LD_{50} values (mouse) were reported for cucurbitacin E with 340 mg/kg body weight, p.o. and for cucurbitacin I 5 mg/kg body weight, p.o. (Reichling and Saller 2002).
Regarding to these toxicological data and the preparations used in the homeopathic treatment the amount of free cucurbitacins in homeopathic mother tinctures has only weak toxicological significance.

3. Experimental

3.1. Apparatus and conditions

HPLC was performed with an Hewlett Packard (HP Waldbronn, Germany) model 1090 liquid chromatograph equipped with an automatic sample injector and diode-array detector. Separation was performed on a Eurospher C_{18} (250 mmx 4 mm i.d; 5 μm) reversed-phase HPLC column with precolumn. The mobil phase was prepared from two solutions: A (acetonitrile) and B (water). The gradient profile was performed 0–35 min linearly from 20% A to 60% B, 35–40 min linearly from 60% to 20%, then held for 5 min. The flow rate was 1 ml min^{-1} at a column temperature of 25 °C. The injection volume was 20 μl, and the detection wavelength was 230 nm.

3.2. Materials

All reagents were of analytical-reagent grade. Solvents were degassed in an ultrasonic bath for 15 min. Cucurbitacin E (Rotichrom HPLC, Carl Roth GmbH, Karlsruhe Germany) and cucurbitacin I (Rotichrom HPLC, Carl Roth GmbH, Karlsruhe Germany) were purchased as reference substances. Standard solutions were prepared by dissolving cucurbitacin E and cucurbitacin I in acetonitrile solution and they were filtered using a 0,45 μm, 13 mm, PTFE (Teflon) (Carl Roth GmbH) filter. β-Glucosidase (from almonds) was purchased from Roth (≥1000 U/mg).

3.3. Plant material

Commercial *Gratiola officinalis* mother tincture prepared according to monograph Gratiola HAB 2000/method 2a from DHU (Lot V2A URT) was used in the experiments.

3.4. Preparation of samples

3.4.1. Determination of aglycones

Mother tincture (3 ml) was extracted with 3 × 10 ml of chloroform. The chloroform extracts were combined, evaporated under reduced pressure and dissolved in acetonitrile. They were filtered using a 0.45 μm, 13 mm, PTFE (Teflon) (Carl Roth GmbH) filter.

3.4.2. Determination of glycosides and aglycones

Mother tincture (1 ml) was mixed with 3.5 ml of 0.1 M sodium acetate buffer, pH 6.0, than added 0.5 ml of 0.1 M sodium acetate buffer solution of β-glucosidase (0.15 mg from 1000 U/mg). The reaction mixture was incubated at 37 °C. After 24 h of incubation, pH was adjusted to 7.0 with NaOH, extracted with 3x10 ml of chloroform. The chloroform extracts were combined, evaporated under reduced pressure and dissolved in acetonitrile. The solution was filtered using a 0.45 μm,13 mm, PTFE filter.

3.4.3. Calibration graph

Five standard solutions containing 15.7 to 250 μg/ml of cucurbitacin E and cucurbitacin I were injected into the column. Linear responses (peak area) were obtained in this range of concentration, the regression equation for cucurbitacin E was $y = 12017x - 37648$ ($r^2 = 0.99$, $n = 5$) and for cucurbitacin I was $y = 12323x - 73051$ ($r^2 = 0.99$, $n = 5$), where y = peak area, x = concentration (μg/ml), r^2 = correlation coefficient and n = number of points on the curve (each representing triplicate injections).

3.4.4. Recovery

To determine the sample recovery, cucurbitacin E standard solution (50 μg/ml) and cucurbitacin I (40 μg/ml) in a 43% (v/v) alcohol solution were extracted and analysed as described above for the mother tincture sample. The mean recovery of the cucurbitacin E content was 95% and of cucurbitacin I 96.5%, respectively.

References

Bauer R, Wagner H (1983) Cucurbitacinhaltige Drogen. Dtsch Apoth Ztg 123: 1313–1321.

Chen JC, Chiu MH, Nie RL, Cordell GA, Qiu SX (2005) Cucurbitacins and cucurbitane glycosides: structures and biological activities. Nat Prod Rep 22: 386–399.

Duncan KL, Duncan MD, Alley MC, Sausville EA (1996) Cucurbitacin E-induced disruption of the action and vimentin cytoskeleton in prostate carcinoma cells. Biochem Pharmacol 52: 1553–1560.

Fuller RW, Cardellina II JH, Cragg GM, Boyd MR (1994) Cucurbitacins: differential cytotoxicity, dereplication and first isolation from *Gonystylus keithii*. J Nat Prod 57: 1442–1445.

German Homeopathic Pharmacopea/Homöopathisches Arzneibuch 2007, Amtliche Ausgabe, Deutscher Apotheker Verlag, Stuttgart

Gmelin R (1967) Wirkstoffanalyse von *Gratiola officinalis* L. Arch Pharm 300: 234–240.

Kreis W (2007) Enzyme bei der Gewinnung von Drogen und der Herstellung von Phytopharmaka. In Hänsel R, Sticher O, Pharmakognosie Phytopharmazie 8th ed., Heidelberg, p. 291

Lavie D, Glotter E (1971) Cucurbitanes, a group of tetracyclic triterpenes. Fortschr Chem Org Naturstoffe 29: 307–362.

Le Men J, Buffard G, Provost J, Tiberghien R, Forgacs P, Lagrange E, Albert O, Aurousseau M (1969) Relation between structure of cucurbitacins and their toxicity and laxative activity. Chimica Therapeutica 4: 459–465.

Müller A, Wichtl M (1979) Zur Frage der Herzwirksamkeit des Gnadenkrautes (Gratiola officinalis L.). Pharm Ztg 124: 1761–1766.

Reichling J, Saller R (2002) *Iberis amara* L. (bitter candytuft) – profile of a medicinal plant. Forsch Komplementärmed Klass Naturheilkd 9: 21–33.

Sturm S, Stuppner H (2000) Analysis of cucurbitacins in medicinal plants by high-pressure liquid chromatography mass spectrometry. Phytochem Anal 11: 121–127.

Wiesenauer M (1996) Homöopathie für Ärzte und Apotheker, Deutscher Apotheker Verlag, Stuttgart.