Qualitative and quantitative analysis of aconitine-type and lipo-alkaloids of Aconitum carmichaelii roots

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ABSTRACT

By optimizing the extraction and analytical conditions, a reliable and precise HPLC method coupled with photodiode array detection (HPLC–DAD) has been developed for the identification and quantification of three major aconitine-type alkaloids (aconitine, mesaconitine, hypaconitine) in the roots of Aconitum carmichaelii Debeaux. The qualitative analysis of the plant material was carried out by LC-APCI-MS. By means of this method, 26 lipo-alkaloids were also identified from the roots of A. carmichaelii. The effect of processing on aconitine-type alkaloids, lipo-alkaloids and pure aconitine was studied. As part of our investigation, two lipo-alkaloids, 14-benzoylaconine-8-palmitate and 14-benzoylaconine-8-linoleate were produced semisynthetically. The COX-1, COX-2 and LTB4 formation inhibitory activity of aconite root extracts and different types of diterpene alkaloids and the toxicity of lipo-alkaloids were also investigated.

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1. Introduction

Preparations of certain Aconitum species native to Asia are indispensable materials in Eastern medicine. The tubers and roots of aconites are applied only after cautious processing (usually boiling) in order to reduce their toxicity. The processed drugs are typically used as painkillers and antirheumatic agents. The unprocessed roots are too toxic for internal use, but are used for external application as anesthetics. In the past few years aconite roots have become increasingly popular in many countries outside China. There have been several cases of poisoning when unprocessed or improperly processed Aconitum preparations were applied internally.

Aconitine-type alkaloids, the main compounds of several species, belong to the most toxic natural products. They are esterified with acetic acid and a benzoic acid at C-8 and C-14, respectively. The high toxicity of aconites is attributed to these aconitine-type alkaloids, and it has been shown, that during processing hydrolysis of the ester groups decreases the toxicity. Aconitum roots also contain the so-called lipo-alkaloids, which are compounds esterified with fatty acids at the C-8 carbon; their pharmacology has not yet been thoroughly investigated. Lipo-alkaloids are more stable than aconitine-type alkaloids. Whilst the latter compounds are decomposed during the processing of the roots, lipo-alkaloids were detected both in unprocessed and in processed roots of Aconitum carmichaelii. Despite their low concentration in the plant material, the pharmacological interest in lipo-alkaloids is further enhanced by the fact, that aconitine-type alkaloids can be converted to lipo-alkaloids by the human intestinal flora.

The most widely used Aconitum species in China is A. carmichaelii Debeaux. Though it is recognized that the toxicity of aconites can be attributed to the aconitine-type diterpene alkaloid content of the plants, in the Chinese Pharmacopoeia the quality control of the unprocessed roots is based on organoleptic and spectrophotometric analysis. The standardization of the processed roots is further enhanced by the fact, that aconitine-type alkaloids can be converted to lipo-alkaloids by the human intestinal flora.
however, the effect of the extraction method on the alkaloid content of the extracts was less extensively examined [15,16].

Our experiments aimed at the qualitative and quantitative analysis of diterpene alkaloids of *A. carmichaelii*. The extraction is the cornerstone of each phytochemical analysis, since only effective methods provide reliable analytical results. Therefore, we investigated the influence of extraction methods on the quantitative and qualitative alkaloid content of *A. carmichaelii* extracts to develop a reliable HPLC–DAD method for the quantification of the major aconitine-type alkaloids, aconitine (1), hypaconitine (2) and mesaconitine (3) (Fig. 1). Additionally, we identified 26 lipo-alkaloids (11–36) from the root extracts by LC-APCI-MS<sup>n</sup>. The effect of processing on aconitine-type alkaloids, lipo-alkaloids and pure aconitine was also studied. The analytical work was supplemented with some pharmacological, toxicological investigation in order to evaluate the role of processing and different types of alkaloids in the anti-inflammatory effect of the aconites. We examined the COX-1, COX-2 and LTB4 formation inhibitory activity of extracts from unprocessed and processed roots, aconitine (1), 14-benzoylaconine (5), aconine (7) and the semisynthetically produced 14-benzoylaconine-8-palmilate (9) and 14-benzoylaconine-8-linolenate (10). The acute toxicity of 9 and 10 was also investigated.

2. Experimental

2.1. Plant material

Unprocessed *A. carmichaelii* roots (one sample) were obtained from a pharmacy in China. The unprocessed and processed dry aconite roots were ground, passed through a 0.315 mm sieve.

2.2. Chemicals, buffer, alkaloid standard

Acetonitrile and methanol were of HPLC grade, water (≥ 18 MΩ) was generated by an EASYPure RF compact ultrapure water system (Barnstead, IA, USA). Ammonium acetate buffer (pH 8.9) was prepared using reagent grade glacial acetic acid and 25% ammonia solution. All chemicals were purchased from Sigma–Aldrich. Aconitine, hypaconitine and mesaconitine was purchased from PhytoLab GmbH, Vestenbergsgreuth, Germany. Linoleic acid and bergapten (99.0%) was purchased from Sigma–Aldrich, Germany, palmitic acid (99.0%) was purchased from Sigma–Aldrich, Germany, palmitic acid from Reanal, Hungary.

The purity of the alkaloids aconitine, hypaconitine and mesaconitine was determined by means of NMR according to the method of van Beek et al. [17]. The method was based on the comparison of the integral of each 8-0Ac signal of the three aconitine-type alkaloids with that of the 5-OMe protons of the internal standard bergapten. The purity of aconitine, hypaconitine and mesaconitine was 98.4%, 84.6% and 83.0%, respectively.

2.3. Preparation of extracts

Extracts from the roots of *A. carmichaelii* were prepared with mixtures of MeOH–H<sub>2</sub>O (3:2, 3:1, 9:1, 1:0) and MeOH–CH<sub>2</sub>Cl<sub>2</sub> (3:1, 1:1, 1:3). 0.5 g ground root was extracted at room temperature three times (15 min each) in an ultrasonic bath with 3 × 15 ml solvent. The combined extracts were filtered, concentrated and diluted to 2.0 ml with MeOH.

Acidic and basic solvents were applied in order to carry out selective extraction of alkaloids and to gain extracts poor in neutral compounds. For acidic extraction, 1% aqueous HCl solution was used. After 15 min sonication with 5.00 ml solvent, the extract was centrifuged and the supernatant was further analyzed. For basic extraction, 0.5 g plant material was wetted with 0.2 ml cc. NH<sub>3</sub> and extracted with 100 ml diethyl ether by sonication (30 min). To prevent boiling of diethyl ether, water was changed regularly to cold water in the ultrasonic bath during sonication. The ether phase was extracted with 3 × 25 ml H<sub>2</sub>O to remove NH<sub>3</sub>. The organic phase was concentrated and diluted with MeOH to 2 ml for further analysis.

The extractions were carried out in triplicate. The final resulting solutions were filtered through a 0.45-µm membrane (Nalgene 4-mm syringe filters with nylon membrane), and 20-µl samples were examined by HPLC–DAD and LC-APCI-MS<sup>n</sup>.

2.4. Processing of the plant material and aconitine

For processing, a method adapted from the Chinese Pharmacopoeia of the People's Republic of China was applied [11]. Intact *A. carmichaelii* roots (5 g) were soaked in 50 ml water for 1 h, then boiled for 5 h under reflux. The processed roots were dried to constant weight at room temperature, ground and sifted (<0.315 mm). 0.5 g plant material was extracted with MeOH–H<sub>2</sub>O 9:1 and prepared for further analysis, as described in chapter 2.3. The water, in which the roots were boiled (boiling water), was evaporated under vacuum and the dry residue was dissolved in 2.0 ml MeOH for further analysis.

In case of aconitine (1), 30 mg pure compound was dissolved in 3 ml MeOH and mixed with 50 ml water and boiled for 5 h. After evaporation, the dry residue was analyzed with LC-APCI-MS<sup>n</sup>. 14-benzoylaconine (5) and aconine (7) were isolated from the reaction mixture by preparative TLC on SiO<sub>2</sub> plates (20 × 20 cm Silica gel 60 F<sub>254</sub>, Merck 5715, layer thickness 0.25 mm) in saturated chamber with toluene–acetone–EtOH–CC. NH<sub>3</sub> 70:50:16:4.5 as mobile phase. The compounds were eluted from the scraped adsorbent with CHCl<sub>3</sub>–MeOH 9:1. The structures of 14-benzoylaconine (5) and aconine (7) were confirmed by comparing their NMR spectra with those in the literature [18,19]. The yield of the method was 18% and 30% for 14-benzoylaconine (5) and aconine (7), respectively.

2.5. Quantitative analysis and method validation

Aconitine (1), hypaconitine (2) and mesaconitine (3) were accurately weighed and dissolved with MeOH to produce stock solutions. Calibration curves were established for each alkaloid taking into account the purity of the compounds, based on seven concentrations (with a range of 12.3–184.5 µg/ml for aconitine (1), 21.1–422.9 µg/ml for hypaconitine (2) and 41.5–829.5 µg/ml for mesaconitine (3)) by diluting the stock solutions with MeOH in appropriate quantities.

To determine the repeatability of the most effective extraction method, 5 samples of unprocessed roots were extracted in parallel with 1% aqueous HCl and analyzed by HPLC–DAD. The same extract prepared with 1% HCl was subjected 5 times to HPLC–DAD analysis on the same day to determine the precision of the analytical method. The aconitine (1), hypaconitine (2) and mesaconitine (3) content of the samples were measured, and the relative standard deviation (RSD) within the measurements was used to evaluate the repeatability and precision.

The recovery of mesaconitine (3), the major diterpene alkaloid was investigated by analysis of root samples with determined alkaloid content spiked with two different known amounts of mesaconitine. The samples were then extracted with 1% HCl and quantified with HPLC–DAD as described earlier. The relative recovery of aconitine (1), hypaconitine (2) and mesaconitine (3) was determined by subjecting the acidic extract to a repeated sonication, centrifugation and filtration as described in Section 2.3, followed by quantification of the alkaloids by HPLC–DAD.
2.6. HPLC–DAD and LC–MS analysis

For HPLC–DAD analysis, a simple and quick method was developed using a Merck Hitachi HPLC system (pump: L-7100 LaChrom; diode array detector: L-7455 LaChrom) on a reversed phase column (Agilent Zorbax Eclipse XDB-C8 4.6 mm × 150 mm, 5 μm). Separation of alkaloids was carried out using a gradient system of 10 mM ammonium acetate buffer (pH 8.9) and MeOH (0 min: buffer–MeOH 60:40; 35 min: buffer–MeOH 5:95; 36 min: buffer–MeOH 5:95; 42 min: buffer–MeOH 60:40; 45 min: buffer–MeOH 60:40) at a flow rate of 1 ml/min. The alkaloids were detected at 233 nm.

LC–MS analysis was carried out on a Thermo Finnigan Surveyor liquid chromatograph interfaced with a LCQ™ Deca XP-PLUS mass detector in the APCI positive mode. The vaporizer temperature was 400 °C, capillary temperature 350 °C, discharge current 5 mA, capillary voltage 25 V, tube lens offset −15 V. Sheath gas flow was 55, and auxiliary gas flow 15 arbitrary units. The chromatographic conditions (stationary phase, mobile phase) were the same as in the case of HPLC–DAD analysis.

2.7. Preparation of 14-benzoylaconine-8-palmitate (9) and 14-benzoylaconine-8-linoleate (10)

14-Benzoylaconine-8-palmitate (9) was produced semisynthetically according to the modified method of Bai et al. [20]. 30 mg aconitine (1) and 60 mg palmitic acid were mixed and heated in an oil bath (110 °C) for 3 h under vacuum (10 mbar). The reaction mixture was dissolved in CHCl₃–MeOH 1:1 and purified by gel filtration. Gel filtration chromatography was performed on Sephadex LH-20 (25–100 μm, Pharmacia Fine Chemicals) using CHCl₃–MeOH 1:1 as eluent. The yield was 39.4%.

14-Benzoylaconine-8-linoleate (10) was prepared of 30 mg aconitine (1) and 60 mg linoleic acid according to the method described above. The compound was purified by preparative TLC on SiO₂ plates (20 × 20 cm Silica gel 60 F254, Merck 5715) with toluene–acetone–EtOH–cc:NH₃ 70:50:16:4.5 as mobile phase. The compound was eluted from the scraped adsorbent with CHCl₃–MeOH 9:1. The yield was 35%.

Compounds 9 and 10 were obtained as yellowish oils. As byproduct, in both cases pyroaconitine was identified.

2.8. Structure elucidation of 9 and 10 by NMR

14-Benzoylaconine-8-palmitate (9): 1H NMR (600 MHz, CDCl₃) δ (ppm) 3.13 t (1H, J = 6.6 Hz, H-1), 2.38 m (1H, H-2a), 1.96 m (1H, H2b), 3.80 dd (1H, J = 9.3 Hz and 4.9 Hz, H-3), 2.15 m (1H, H-5), 4.04 d (1H, J = 6.6 Hz, H-6), 2.85 s (1H, H-7), 2.91 m (1H, H-9), 2.13 m (1H, H-10), 2.69 m (1H, H-12a), 2.13 m (1H, H-12b), 4.86 d (1H, J = 5.0 Hz, H-14), 4.44 dd (1H, J = 5.3 Hz and 2.3 Hz, H-15), 3.34 d (1H, J = 5.4 Hz, H-16), 3.10 s (1H, H-17), 3.61 d (1H, J = 8.9 Hz, H-18a), 3.46 d (1H, J = 8.9 Hz, H-18b), 2.89 d (1H, J = 10.7 Hz, H-19a), 2.36 m (1H, H-19b), 2.74 dq (1H, J = 14.4 Hz and...
2.9. Assays for COX-1, COX-2 and LTB₄ formation inhibitory activity

For examining cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2) and leukotriene B₄ (LTB₄) formation inhibitory activities, extracts and pure compounds were dissolved in absolute EtOH. Extracts were tested at a concentration of 50 μg/ml in the assay mixture, pure compounds at 50 μg/ml in the assay mixture. Each sample was tested in 2 independent experiments in duplicate.

COX-1 and COX-2 inhibition assays were performed in a 96-well-plate format with purified prostaglandin H synthase (PGHS)-1 from ram seminal vesicles for COX-1 and purified PGHS-2 from sheep placental cotyledons for COX-2 (both Cayman Chemical Company, Ann Arbor, USA) as previously described [21]. The concentration of PGE₂, the main arachidonic acid metabolite in this reaction, was determined by a competitive PGE₂ EIA kit (Assay Designs Inc., Ann Arbor, MI, USA).

The bioassay for inhibition of 5-LOX mediated LTB₄ formation was carried out in a 96-well-plate format with stimulated human neutrophile granulocytes isolated from human blood as described by Adams et al. [22] with slight modifications [23].

2.10. Investigation of acute toxicity of 9 and 10

Stock solutions of 10 mg/ml of 14-benzoylaconine-8-palmitate (9) and 14-benzoylaconine-8-linoleate (10) and the positive control aconitine (10) were prepared in dimethylsulfoxide (DMSO) and diluted with phosphate-buffered saline (PBS). Male CFLP mice were treated intravenously with a dosing volume of 100 μl/10 g body weight. The starting dose was 1 mg/kg which was increased or decreased according to the results. After administration of the alkaloids mice were observed for 24 h. The experimental protocol satisfied the Guidelines for Animal Experimentation approved by the Animal Experimentation Committee of the University of Szeged.

3. Results and discussion

3.1. Qualitative analysis of aconitine-type alkaloids

From the crude roots of A. carmichaelii, three major aconitine-type alkaloids, aconitine (1), hyaconitine (2), mesaconitine (3) and one further aconitine-type alkaloid, 10-hydroxy-mesaconitine (4) were identified by LC-APCI-MSⁿ by comparing the [M+H]⁺ m/z and fragmentation pattern of the compounds with literature data [9]. These alkaloids were detected in the retention time period of 18–24.5 min. In the full-scan mass spectrum of the extracts the protonated ions of aconitine-type alkaloids were observed in the m/z 600–700 region, and signals with m/z 800–900 correspond to lipo-alkaloids (Fig. 2). The major fragment ion in the MS² spectra of the aconitine-type diterpene alkaloids was [M+H-AcOH]⁺ (a neutral loss of 60 Da), since acetic acid is connected to C-8, which is
active site in the norditerpene skeleton (Table 1) [9]. In addition
to the major fragments [M+H-AcOH]+, some low-abundance peaks
were also detected as result of neutral losses of 18 Da (H2O), 28 Da
(CO), 32 Da (CH3OH) and 60 Da (AcOH). In MS3, the major fragments
provide a base peak [M+H-AcOH-CH3OH-CO]+ due to the loss of
CH3OH plus CO, except for hypaconitine (2), where a neutral loss of
32 Da, corresponding to CH3OH, was observed [9].

3.2. Analysis of the effect of processing on aconitine-type alkaloids

The alkaloid composition of the processed roots and the alkaloid
content of the water in which the roots were processed (boiling water) was analyzed by LC–APCI-MSn. In the processed roots, no
aconitine-type alkaloids could be detected. In the boiling water neither
aconitine-type diterpene alkaloids, nor their unesterified deriva-
tives were detectable.

The LC–APCI-MSn analysis of the reaction mixture gained after
processing pure aconitine (1) resulted in the identification of the
less toxic 14-benzoylaconine (5) and aconitine (7) (data not shown).
This was reassured by the NMR analysis of the purified compounds
5 and 7 (see Section 2.4). In the “processed” sample no aconitine
(1) could be detected.

3.3. Qualitative analysis of lipo-alkaloids

The 90% MeOH extract of unprocessed roots, 90% MeOH extracts of
processed roots, acidic and basic extracts of unprocessed roots
and the boiling water were analyzed for their lipo-alkaloid content.
In the LC–MS chromatograms, the signals of lipo-alkaloids were
detected with retention times of 35–40 min.

In previous studies, lipo-alkaloids were identified in this range
by electrospray ionization (ESI) LC–MS by determining their
quasimolecular peaks [M+H]+ and analyzing their fragmentation
[8,9,24,25]. In the course of our investigations, we analyzed the
extract of the roots of A. carmichaelii applying an LC–APCI-MSn
method. The MSn analysis provided diagnostic information for
structure determination of these alkaloids. Fatty acids are relatively
easily eliminated by electrospray or chemical ionization from C–8,
therefore the major fragment ion in the MS2 spectra of these
alkaloids was [M+H-RCOOH]+ (daughter ion) [9]. The neutral loss
denotes the fatty acid, the major fragment ion determines the
diterpene core of the molecule (Table 2). In the MS3 spectra of the
daughter ions at m/z 556 and 572, the most abundant signals were those of the protonated ions [M+H-AcOH-CH3OH-
CO]+, however in case of m/z 556 a neutral loss of 32 Da provided the
base peak. The spectral characteristics of the daughter ions at m/z
556, 572, 586 and 602 are identical to those published for hypaconitine, deoxyaconitine, mesaconitine, aconitine, 10-
hydroxy-mesaconitine and 10-hydroxy-aconitine, which led to the
identification of 14-benzoylhyponaconitine, 14-benzoyldeoxyaconitine,
14-benzoylmesaconine, 14-benzoylhyponaconitine, 10–OH-14-
benzoylmesaconine and 10-hydroxy-14-benzoylmesaconine core
in the molecules, respectively [8,9]. As a result of our investigations 26 lipo-alkaloids (11–36)
were identified from the extracts of A. carmichaelii roots, 4 of which (14-benzoylhyponaconitine–8–nonadecenoate
and 14-benzoylmesaconine–8–linoleate
As a result of our investigations 26 lipo-alkaloids (11–36)
were identified from the extracts of A. carmichaelii roots, 4 of which (14-benzoylhyponaconitine–8–nonadecenoate
and 14-benzoylmesaconine–8–linoleate

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Aconitine (1)</th>
<th>Hypaconitine (2)</th>
<th>Mesaconitine (3)</th>
<th>10-Hydroxy-mesaconitine (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[M+H-18]+</td>
<td>628 (&lt;1)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>[M+H-32]+</td>
<td>614 (1)</td>
<td>584 (8)</td>
<td>614 (1)</td>
<td>630 (1)</td>
</tr>
<tr>
<td>[M+H-32-18]+</td>
<td>596 (4)</td>
<td>–</td>
<td>582 (7)</td>
<td>598 (14)</td>
</tr>
<tr>
<td>[M+H-60]+</td>
<td>586 (100)</td>
<td>556 (100)</td>
<td>572 (100)</td>
<td>588 (100)</td>
</tr>
<tr>
<td>[M+H-60-18]+</td>
<td>568 (1)</td>
<td>–</td>
<td>554 (2)</td>
<td>570 (4)</td>
</tr>
<tr>
<td>[M+H-60-32]+</td>
<td>554 (7)</td>
<td>524 (16)</td>
<td>540 (7)</td>
<td>556 (9)</td>
</tr>
<tr>
<td>[M+H-60-32-18]</td>
<td>536 (3)</td>
<td>–</td>
<td>522 (4)</td>
<td>538 (8)</td>
</tr>
<tr>
<td>[M+H-60-32-28]</td>
<td>526 (8)</td>
<td>496 (3)</td>
<td>512 (6)</td>
<td>528 (6)</td>
</tr>
<tr>
<td>[M+H-60-32-28]+</td>
<td>522 (&lt;1)</td>
<td>492 (2)</td>
<td>508 (&lt;1)</td>
<td>524 (1)</td>
</tr>
</tbody>
</table>

Fig. 3. HPLC profile of unprocessed (I) and processed (II) Aconitum carmichaelii roots
(mesaconitine (MA), hypaconitine (HA) and aconitine (AC)).
14-Hydroxy-14-benzoylaconine-8-linoleate (10-Hydroxy-14-benzoylmesaconine-8-linoleate (10-Hydroxy-14-benzoylmesaconine-8-linolenate (14-Benzoylaconine-8-oleate (14-Benzoylaconine-8-linoleate (14-Benzoylmesaconine-8-nonadecenoate (14-Benzoylmesaconine-8-oleate (14-Benzoylmesaconine-8-linoleate (14-Benzoylmesaconine-8-palmitate (14-Benzoylmesaconine-8-palmitoleate (14-Benzoylmesaconine-8-pentadecanoate (14-Benzoylmesaconine-8-pentadecenoate (14-Benzoyldeoxyaconine-9-linoleate (14-Benzoylhypaconine-8-nonadecenoate (14-Benzoylhypaconine-8-linoleate (14-Benzoylhypaconine-8-oleate (3-mesaconitine (1-aconitine-type alkaloids, aconitine (3.4. Quantitative analysis

Peaks in the chromatograms (Fig. 3) were identified by comparing the considerable chemical stability of this type of compounds. The high variability of lipo-alkaloids in different extracts can be in the processed roots and the boiling water. This fact refers to the non-polar character in lipo-alkaloids. This can be explained by the non-polar character of the solvent. Interestingly, lipo-alkaloids could also be detected in the processed roots and the boiling water. This fact refers to the considerable chemical stability of this type of compounds. The high variability of lipo-alkaloids in different extracts can be the result of ester-exchange reactions during the extraction or processing of the drug [8].

3.4. Quantitative analysis

In the course of our experiments, we quantified the main aconitine-type alkaloids, aconitine (1), hypaconitine (2) and mesaconitine (3) by HPLC–DAD in A. carmichaelii root extracts. Peaks in the chromatograms (Fig. 3) were identified by comparing the retention time with those of the standards and by LC-APCI-MSn. Linear regression analysis for aconitine (1), hypaconitine (2) and mesaconitine (3) was performed by the external standard method. The regression equation for aconitine was

\[ y = 4513x - 156 \times 10^2 + 812 \]  

The most effective extraction in neutral medium was carried out with the application of the mixture MeOH–H2O 9:1 (Fig. 5). The method using diethyl ether + NH3 for extraction was taken from the Swiss Pharmacopoeia [28]. This extraction method provided an extract free of polar neutral compounds, however, the quantity of aconitine-type alkaloids was not higher than in the case of extracts prepared in neutral medium.

Acids protonate the diterpene alkaloids, hence by extraction with 1% HCl the hydrochloride salts could be extracted with water. This simple method proved to be the most effective one (Fig. 5), and had almost double effectiveness than the most effective method using neutral solvent (MeOH–H2O 9:1).

The acidic extraction and HPLC–DAD analysis method developed by us has good repeatability, precision and (Table 3), therefore it may be the basis for the quantification of the diterpene alkaloid content of crude Aconitum roots. The recovery of mesaconitine (3) in case of spiking with 200 mg was 82.6%, in case of spiking with 400 mg 84.9%. To assess the effect of the extraction and purification method on the alkaloid content, the acidic extracts were subjected repeatedly to sonication, centrifugation and filtration. The mesaconitine (3) content of the samples was reduced to 82.9% (relative recovery).

Since esterified diterpene alkaloids are hydrolyzed not only during boiling, but also as a result of changing the pH, we analyzed the stability of the major aconitine-type alkaloids in the extract by HPLC–DAD using the method described in Section 2.6. The amount of the alkaloids exceeded the 95% of their initial quantity after 21 days storage at +4 °C.

3.5. COX-1, COX-2 and LTB4 formation inhibitory activities of unprocessed and processed roots and compounds 1, 5, 7, 9 and 10

The extracts of A. carmichaelii roots showed moderate COX-2 inhibitory activity, and as the result of processing this activity slightly increased (Table 4). This effect may play a role in the medic-

### Table 2
Lipo-alkaloids identified by APCI-MSn in the different extracts of the roots of A. carmichaelii.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fragment (m/z)</th>
<th>Neutral loss (Da)</th>
<th>Precursor ion</th>
<th>Unprocessed root extract</th>
<th>Processed root extract</th>
<th>Boiling water</th>
<th>Alkaline extract</th>
<th>Acidic extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-Benzoylprofaconine-8-palmitate (11)</td>
<td>556</td>
<td>256</td>
<td>812</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-Benzoylprofaconine-8-linolenate (12)</td>
<td>278</td>
<td>834</td>
<td>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-Benzoylprofaconine-8-linoleate (13)</td>
<td>280</td>
<td>836</td>
<td>*</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-Benzoylprofaconine-8-oleate (14)</td>
<td>282</td>
<td>838</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-Benzoylprofaconine-8-nonadecanoate (15)</td>
<td>296</td>
<td>852</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-Benzoyldeoxyaconine-8-palmitate (16)</td>
<td>570</td>
<td>256</td>
<td>826</td>
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<td>+</td>
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<tr>
<td>14-Benzoyldeoxyaconine-8-linoleate (17)</td>
<td>280</td>
<td>850</td>
<td>*</td>
<td>+</td>
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<tr>
<td>14-Benzoylmesaconine-8-pentadecanoate (18)</td>
<td>572</td>
<td>240</td>
<td>812</td>
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<tr>
<td>14-Benzoylmesaconine-8-pentadecanolate (19)</td>
<td>242</td>
<td>814</td>
<td>+</td>
<td>+</td>
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<tr>
<td>14-Benzoylmesaconine-8-palmitolate (20)</td>
<td>254</td>
<td>826</td>
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<td>828</td>
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<tr>
<td>14-Benzoylmesaconine-8-linolenate (23)</td>
<td>278</td>
<td>850</td>
<td>+</td>
<td>+</td>
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<td>14-Benzoylmesaconine-8-linoleate (23)</td>
<td>280</td>
<td>852</td>
<td>+</td>
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<tr>
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<td>282</td>
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<td>+</td>
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<tr>
<td>14-Benzoylmesaconine-8-stearate (25)</td>
<td>284</td>
<td>856</td>
<td>+</td>
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<tr>
<td>14-Benzoylmesaconine-8-nonadecadienoate (26)</td>
<td>294</td>
<td>866</td>
<td>+</td>
<td>+</td>
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<tr>
<td>14-Benzoylmesaconine-8-nonadecenoate (27)</td>
<td>296</td>
<td>868</td>
<td>+</td>
<td>+</td>
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<tr>
<td>14-Benzoylmesaconine-8-eicosenoate (28)</td>
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<tr>
<td>14-Benzoylmesaconine-8-eicosanoate (29)</td>
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<tr>
<td>14-Benzoylmesaconine-8-linolenate (30)</td>
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<td>864</td>
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<td>14-Benzoylmesaconine-8-oleate (32)</td>
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<td>+</td>
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<td></td>
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<tr>
<td>10-Hydroxy-14-benzylmesaconine-8-linolenate (33)</td>
<td>588</td>
<td>278</td>
<td>866</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-Hydroxy-14-benzylmesaconine-8-linoleate (34)</td>
<td>280</td>
<td>868</td>
<td>+</td>
<td></td>
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<td></td>
</tr>
<tr>
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<td>282</td>
<td>870</td>
<td>+</td>
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</tr>
<tr>
<td>10-Hydroxy-14-benzylmesaconine-8-linoleate (36)</td>
<td>602</td>
<td>280</td>
<td>882</td>
<td>+</td>
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</tr>
</tbody>
</table>
inal application of the drug, beside the pyro-type alkaloids that are
formed during processing [29]. From the tested pure compounds,
only 14-benzoylaconine-8-linoleate (10) markedly inhibited COX-
2. The different COX-2 inhibitory activities of the lipo-alkaloids 9
and 10 may be caused by the structure of the esterifying fatty acid.

Linoleic acid significantly inhibits COX-2, however, palmitic acid
lacks this activity [30]. Neither the tested extracts, nor the alka-
loids 1, 5, 7, 9 and 10 inhibited the COX-1 enzyme and the 5-LOX
mediated formation of LTB4.

3.6. Acute toxicity of 9 and 10

14-Benzoylaconine-8-palmitate (9) and 14-benzoylaconine-8-
linoleate (10) resulted in no observable reactions given in the dose
of 1 mg/kg intravenously for a group of 5 mice. The dose was then
increased to 3 and 10 mg/kg but all animals (5 in a group) survived.

Table 3
Validation characteristics of the extraction method carried out with 1% HCl coupled
with HPLC–DAD analysis.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precision (RSD%)</th>
<th>Repeatability (RSD%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aconitine (1)</td>
<td>1.48</td>
<td>1.38</td>
</tr>
<tr>
<td>Hypaconitine (2)</td>
<td>1.05</td>
<td>0.33</td>
</tr>
<tr>
<td>Mesaconitine (3)</td>
<td>1.76</td>
<td>0.26</td>
</tr>
</tbody>
</table>
Table 4 Screening of Aconitum carmichaelii root extracts (50 μg/ml) and pure alkaloids (50 μM) for COX-1, COX-2 and LT formation inhibitory activity.

<table>
<thead>
<tr>
<th>Sample</th>
<th>% COX-1 inhibition ± SD</th>
<th>% COX-2 inhibition ± SD</th>
<th>% LT formation inhibition ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unprocessed aconite root extract</td>
<td>7.21 ± 7.64</td>
<td>47.86 ± 2.60</td>
<td>35.79 ± 6.85</td>
</tr>
<tr>
<td>Processed aconite root extract</td>
<td>24.42 ± 2.47</td>
<td>65.45 ± 3.26</td>
<td>35.38 ± 5.96</td>
</tr>
<tr>
<td>Aconitine (1)</td>
<td>−4.25 ± 4.64</td>
<td>9.96 ± 8.31</td>
<td>−0.39 ± 6.97</td>
</tr>
<tr>
<td>14-Benzoylaconine (5)</td>
<td>−2.53 ± 6.21</td>
<td>14.77 ± 5.30</td>
<td>−6.03 ± 6.52</td>
</tr>
<tr>
<td>Aconine (7)</td>
<td>2.92 ± 7.81</td>
<td>12.27 ± 4.16</td>
<td>−5.57 ± 9.55</td>
</tr>
<tr>
<td>14-Benzoylaconine-8-palmitate (9)</td>
<td>1.55 ± 2.75</td>
<td>4.15 ± 4.92</td>
<td>25.81 ± 5.77</td>
</tr>
<tr>
<td>14-Benzoylaconine-8-linoleate (10)</td>
<td>2.58 ± 6.70</td>
<td>72.22 ± 3.19</td>
<td>19.61 ± 6.80</td>
</tr>
</tbody>
</table>

All mice (3 in a group) treated with 1 mg/kg aconitine (1) died immediately exhibiting excitatory symptoms. Another 3 animals treated with 0.3 mg/kg aconitine (1) also died in the same way. After the administration of 0.1 mg/kg all out of 4 mice survived without toxic reactions.

As far as the acute toxicity of 14-benzoylaconine-8-palmitate (9) and 14-benzoylaconine-8-linoleate (10) is concerned, they are substantially less toxic than aconitine (1) as evidenced by the difference in the lethal intravenous single doses (more than 10 mg/kg for lipo-alkaloids versus 0.3 mg/kg for aconitine (1)).

4. Conclusions

As a result of our experiments, a highly effective and reliable extraction and analytical method was developed for the analysis of aconitine-type alkaloids in Aconitum roots. In the unprocessed sample examined by us, mesaconitine (3) was the main alkaloid, which highlights the importance of the standardization of aconite drugs based on the determination of the quantity of the major aconitine-type alkaloids. However, in the processed roots no aconitine-type alkaloids were detected, which confirms the effectiveness and rationale of the processing method. Lipo-alkaloids were identified both in the processed and unprocessed drugs, which refers to their considerable stability and the possibility of ester-exchange reactions during boiling. The increased number of lipo-alkaloids after processing suggested that during the processing trans-esterification of aconitine-type alkaloids may take place. The acute toxicity of the lipo-alkaloids examined by us was by several orders of magnitude lower than that of aconitine (1). This, together with the moderate COX-2 inhibitory activity of 14-benzoylaconine-linoleate (10) raises the issues of the potential role of lipo-alkaloids in the anti-inflammatory effect of processed Aconitum roots.

Our results underline the necessity of quantifying all the major aconitine-type alkaloids in aconite roots to assess the toxicity of the crude herbal material. In addition, for sample preparation the most effective extraction method should be applied to ensure reliable results. The discovery of the first COX-2 inhibiting lipo-alkaloid may open new ways in the pharmacological investigation of Aconitum species.

Acknowledgements

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References