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Improved detection of alkaloids in crude extracts applying capillary electrophoresis with field amplified sample injection

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Abstract

A simple and effective method for the sensitive detection of alkaloids in crude plant extracts applying capillary electrophoresis with field amplified sample injection (FASI) is described. This method was compared with normal pressure injection for the determination of alkaloids in methanolic extracts from roots of *Berberis vulgaris* L. (*Berberidaceae*) and *Hydrastis canadensis* L. (*Ranunculaceae*) using a 1:1 mixture of 200 m*M* ammonium acetate at pH 3.1 and methanol. By introducing a short plug of 70% methanol (v/v) before electrokinetic injection with 16 kV for 8 s the concentration sensitivity was 1000-times higher compared to hydrodynamic injection for 1 s. No difference between both injection methods for selectivity and resolution of the obtained electropherograms was found. The influence of voltage and injection time on the introduced sample amount was investigated using a mixture of berberine and chelidonine as model substances.

Keywords: Field amplified sample injection; Injection methods; Alkaloids

1. Introduction

The widely used roots of *Berberis vulgaris* L. were traditionally applied for the treatment of liver and gall diseases but also provided a source of pharmacologically interesting protoberberine alkaloids, for example, palmatine (**1**), jatrorrhizine (**2**) or the main alkaloid berberine (**3**) [1]. This quaternary alkaloid is also present in the rhizome and in roots of *Hydrastis canadensis* L. in concentrations of 2 to 3%, without the existence of other quaternary protoberberines and thus represents a useful source for the isolation of berberine (**3**). The extracts of this plant are also medicinally used because of the uterine hemostatic and antiseptic properties of hydrastine (**4**), hydrastinine (**5**) and berberine (**3**) [2]. On account of the great interest in these crude drugs different separation methods including thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) were applied for their alkaloid determination in the past [3,4].

Capillary electrophoresis (CE) has gained widespread interest as a favourable technique for the determination of pharmacologically interesting compounds in biological matrices such as plants and biological fluids like urine or blood [5,6]. The most attractive advantages of CE are rapidity of the method, small sample amounts (nl) required and a strictly limited solvent waste. Additionally, the coupling of CE with mass spectrometry (MS) leads to

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excellent identification of pharmaceuticals and natural products in standard mixtures [7–9] as well as in raw extracts [10–12]. However, the reduced concentration sensitivity of CE compared to HPLC [5] is a crucial point for analysis of substances occurring in very low concentrations. Some strategies were developed to overcome this problem. On the one hand on-line preconcentration techniques like stacking [13], isotachophoresis [14] or electrokinetic injection [15] were applied. On the other hand, highly sensitive detection methods like laser-induced fluorescence (LIF) detection [16] or finally the most promising combination of LIF detection and electrokinetic injection were described. This combination allowed the identification of anthracycline antibiotics in human plasma with a detection limit of 125 to 250 pg ml^{-1} [17]. Although electrokinetic sample introduction is often used for proteins and preferentially for nucleotides, the method was only sparingly applied for the analysis of natural products. The sensitive detection of opiates in urine following electrokinetic injection proved to be a useful technique for the identification of drugs of abuse in body fluids [18]. In addition, CE of pilocarpine and its *trans* epimer isopilocarpine [19] was successfully applied to the analysis of pilocarpine in eyedrops using this method of sample loading. But no work is reported so far concerning CE of complex alkaloid mixtures, which are present in crude extracts applying electrokinetic injection for on-line preconcentration.

Recently we developed an efficient buffer system for capillary zone electrophoresis (CZE) representing a general approach to the analysis of different alkaloid classes using on-line UV- and mass-detection [9]. Because of its overall applicability for various groups of alkaloids, the method was also applied to the analysis of raw extracts from a range of medicinal plants [11,12]. The limited sensitivity of UV detection prompted us to investigate the conditions for an on-line preconcentration via electrokinetic injection.

Therefore we used two alkaloids namely, berberine (**3**) and chelidonine (**6**) for optimisation of injection conditions and a methanolic extract of the roots from *B. vulgaris* and *H. canadensis* in order to compare electrokinetic injection with normal pressure injection. We succeeded in the alkaloid determination of the plant extracts with much higher sensitivity by applying our former developed buffer system [9] with slight modifications.

2. Experimental

2.1. Chemicals

HPLC-grade methanol, ammonium acetate and sodium hydroxide (analytical-reagent grade) were used as supplied by Merck (Darmstadt, Germany). Acetic acid was from AppliChem (Darmstadt, Germany). Canadine, columbamine and jatrorrhizine were kindly provided by Prof. Dr. M.H. Zenk (Munich, Germany). Berberine chloride, chelidonine, hydrastine, hydrastinine hydrochloride, palmatine chloride and tryptamine hydrochloride were purchased from Sigma (Deisenhofen, Germany).

2.2. Sample preparation

2.2.1. Standards

All stock solutions were prepared by using 70% MeOH (v/v) and diluted to the final concentration with the same solvent unless otherwise stated. For hydrodynamic injection of berberine chloride, a stock solution of 1 mg ml^{-1} was prepared and diluted to a concentration of 100 μg ml^{-1}. If injection was performed electrokinetically, a mixture of 1 mg ml^{-1} berberine chloride and 1 mg ml^{-1} chelidonine was diluted to a final concentration of 10 μg ml^{-1}. For three different samples of hydrastine (10 μg ml^{-1}) a stock solution of 10 mg in 100 ml MeOH was diluted ten-times with MeOH, 70% MeOH (v/v) and finally with acetic acid–70% MeOH (v/v) (1:10) mixture.

2.2.2. Extracts

The powdered roots of *B. vulgaris* and *H. canadensis* (5 g each) were extracted with 200 ml MeOH for 12 h in a Soxhlet apparatus. After filtration through cotton wool, the volume was made up to 200 ml, and the deep orange coloured solutions were stored in the dark at room temperature. For pressure injection 100 μl of crude extract was centrifuged at 20 000 *g* for 5 min and the resulting liquid was introduced into the capillary without

further pretreatment. When electromigration was used for sample injection, 100 μl of the extract was diluted to 100 ml with 70% MeOH (v/v) and applied to CZE analysis after centrifugation as mentioned above. In the case of *H. canadensis* an additional 1:100 dilution of extract was prepared by adding 70% MeOH (v/v).

2.3. Instrumentation

A Bio-Rad BioFocus 3000 apparatus (Munich, Germany) equipped with a fast scanning detector and a liquid cooling system for the capillary was used. CE was carried out in a 55 cm (50 cm to detector)× 50 μm I.D. fused-silica column (Polymicro Technologies, Phoenix, AZ, USA). Before starting with analyses the capillary was flushed with 1 *M* sodium hydroxide for 10 min, followed by water (5 min) and 15 min with running buffer. Between runs the capillary was purged with water for 1 min, 2 min with 1 *M* NaOH, followed by 1 min with water and finally 3 min with buffer. If not in use, the capillary was stored in water overnight. The temperature of the sample carousel was maintained at 15°C. Hydrodynamic sample introduction was performed by using a pressure of 345 mbar for 1 s at the capillary inlet corresponding to a sample volume of ca. 7 nl. For FASI a voltage of 16 kV and an injection time of 8 s was applied after preinjection of 70% MeOH (v/v) as stated for hydrodynamic sample introduction. The electrolyte consisted of a 1:1 mixture of 200 m*M* ammonium acetate adjusted to pH 3.1 with acetic acid and MeOH [20]. Prior to use the buffer was passed through a nylon filter (0.45 μm, Macherey–Nagel, Düren, Germany) and degassed for 10 min in an ultrasonic bath.

2.4. Capillary electrophoresis

The separation of alkaloids from *Cortex Berberidis Radicis* was done at a wavelength of 240 nm with a running voltage of 18 kV and a capillary temperature of 15°C. For analysis of *Rhizoma Hydrastis* a voltage of 20 kV instead of 18 kV was used and the temperature was raised to 25°C whereas the detection wavelength was also set to 240 nm.

3. Results and discussion

3.1. Development of CZE conditions for alkaloids

For CZE analysis of alkaloids from *H. canadensis* and *B. vulgaris* our previously developed buffer system [9] was applied. We obtained complete separation of *Hydrastis* alkaloids in less than 25 min using 20 kV and a capillary temperature of 25°C, but only insufficient resolution of the alkaloids from *B. vulgaris* was reached, especially jatrorrhizine (**2**) and columbamine (**7**) (structures of alkaloids are given in Fig. 1). Because this electrolyte contains acetonitrile, its viscosity is reduced. This leads to a higher electroendoosmotic flow [21] compared to buffer systems without organic solvent or buffers consisting of water–alcohol mixtures. In order to achieve excellent resolution of alkaloids present in both extracts we tested different mixtures of 200 m*M* ammonium acetate (pH 3.1) with MeOH in the range of 5–70%. Concentrations between 5 and 40% MeOH gave insufficient selectivity whereas higher amounts of alcohol resulted in much longer migration times. A successful separation of alkaloids was obtained by preparing a 1:1 dilution of 200 m*M* ammonium acetate (pH 3.1) with MeOH providing good selectivity and resolution of substances in less than 35 min.

3.2. Development of injection parameters for FASI

To investigate the influence of different injection parameters, for example, time and voltage on the introduced sample amount, a mixture of berberine chloride and chelidonine (**6**) in 70% MeOH (v/v) was used for FASI. This injection technique first described by Haglund and Tiselius [22] provides a strongly improved concentration sensitivity if samples are prepared in a medium of low conductivity and injected electrokinetically. A modification of this procedure was reported by Chien and Burgi [23], who introduced a short plug of water into the capillary before the sample was injected. By applying this method for the analysis of amino acid derivatives, a several hundred-fold enhancement in the injected amount could be confirmed experimentally [23]. For the analysis of basic antimalarial drugs in urine [24] the sample was prepared in

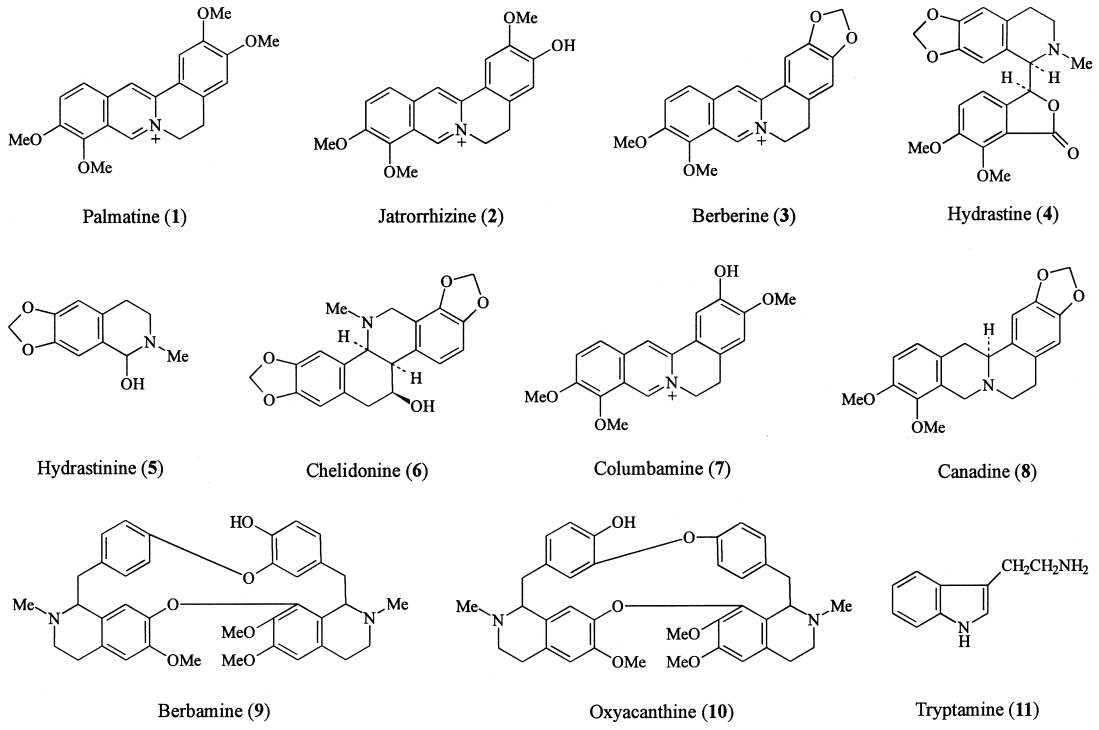

Fig. 1. Names and chemical formulas of berberis/hydrastis alkaloids and tryptamine.

MeOH and injected electrokinetically. Again this procedure provided an improved sensitivity compared to hydrodynamic injection [25].

As shown in Fig. 2 the peak heights of both substances increased parallel to the applied voltage. Although high voltages are very effective for a strong preconcentration of analytes, their use are disadvantageous for sample composition through Joule heating or contamination by electrochemical reaction products as mentioned in Ref. [26]. If the applied injection time exceeded 16 s, the peak height of chelidonine (**6**) decreased slightly, while berberine (**3**) showed a strong reduction in peak height (Fig. 3). Since analyte ions get through the short solvent plug into the running buffer if long injecting times are applied, the sample zone becomes disperse thus resulting in an incomplete stacking process. In fact, this corresponds to the higher electrophoretic mobility of the quaternary berberine (**3**) compared to that of the less basic chelidonine (**6**), which does not migrate into the buffer zone due to its lower electrophoretic mobility.

The introduction of a short plug of 70% MeOH

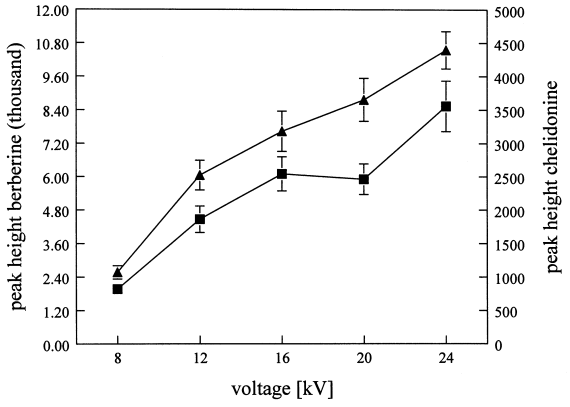

Fig. 2. Influence of the applied injection voltage on the electrokinetically injected amount of berberine (**3**) and chelidonine (**6**). Conditions: injection voltage, 8–24 kV (+ to −); injection time, 4 s; running voltage, 20 kV (+ to −); 25°C; UV detection at 240 nm; buffer: 200 m*M* ammonium acetate pH 3.1–MeOH (1:1, v/v); (▲) berberine (**3**), (■) chelidonine (**6**), concentration: 10 μg ml^{-1} each; mean values±S.D., $n=5$; (for capillary dimensions and sample preparation see Section 2.2 Section 2.3).

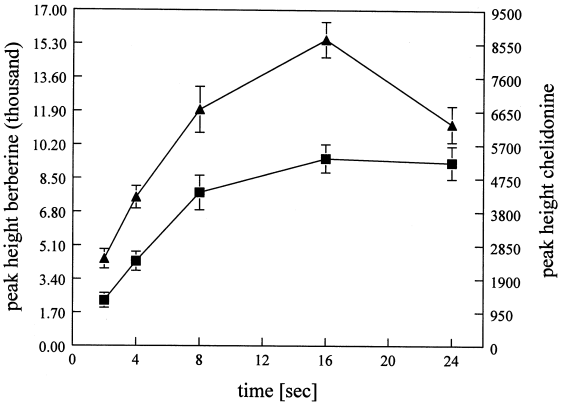

Fig. 3. Influence of the applied injection time on the electrokinetically injected amount of berberine (**3**) and chelidonine (**6**). Conditions: injection voltage, 12 kV (+ to −); injection time, 2–24 s; running voltage, 20 kV (+ to −); 25°C; UV detection at 240 nm; buffer: 200 m*M* ammonium acetate pH 3.1–MeOH (1:1, v/v); (▲) berberine (**3**), (■) chelidonine (**6**), concentration: 10 μg ml^{-1} each; mean values±S.D., $n=5$; (for capillary dimensions and sample preparation see Sections 2.2 and 2.3).

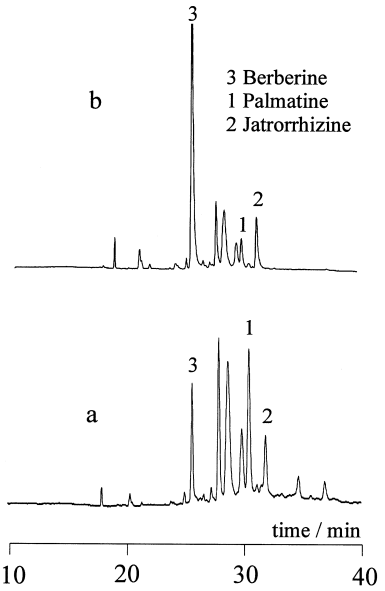

Fig. 4. Normalised electropherograms of a MeOH extract from the roots of *B. vulgaris* obtained after electrokinetic injection with different combinations of time and voltage. Conditions: (a) electrokinetic injection with 24 kV and 16 s (+ to −) after preliminary pressure injection of 70% MeOH (v/v) for 1 s; (b) electrokinetic injection with 16 kV and 16 s (+ to −) after preliminary pressure injection of 70% MeOH (v/v) for 1 s; running voltage 18 kV (+ to −), 15°C, UV detection at 240 nm; buffer: 200 m*M* ammonium acetate pH 3.1–MeOH (1:1, v/v); (for capillary dimensions and sample preparation see Sections 2.2 and 2.3).

(v/v) before starting with injection proved to be effective in two ways. First, this method reduced the relative standard deviation of determined peak heights from 6.1% to 2.2%, and second, the injected amount of alkaloids showed a 1.5-fold increase. A voltage of 24 kV and an injection time of 16 s provided a maximum concentration sensitivity for analysis of the standard mixture containing berberine (**3**) and chelidonine (**6**) as shown in Figs. 2 and 3. But for the crude extracts the use of voltages above 16 kV and injection times higher than 8 s did not improve the preconcentration of the alkaloids.

For analysis of extracts of *B. vulgaris* we tested four different combinations of injection voltage and injection time. The application of 24 kV was effective only in combination with long injection times between 12 and 16 s. For example no peaks were obtained using 24 kV and 8 s. By using 24 kV and 16 s (Fig. 4a) the resulting electropherogram strongly differed from that obtained through pressure injection (Fig. 5b). The alkaloids jatrorrhizine (**2**) and berberine (**3**) show a remarkable decrease in peak height compared to other substances like palmatine (**1**). Moreover there is no improvement in concentration sensitivity as expected from the results achieved for berberine (**3**) and chelidonine (**6**). Also the use of longer injection times, e.g., 16 s together with a voltage of 16 kV did not enhance the detectability of compounds (Fig. 4b). The higher injection voltage of 24 kV (Fig. 4a) instead of 16 kV (Fig. 4b) results in a much higher Joule heating. Consequently this leads to temperature gradients along the capillary wall which cause a strong adsorption of the alkaloids [27,28]. Finally, changes in peak heights and migration times occur (Fig. 4). The additional peaks at about 35 and 37 min are most probably artefacts arising from degradation of substances e.g., by thermal decomposition or through electrochemical reactions [26]. Therefore we used 16 kV and 8 s for analysis of the crude extracts (Fig. 5a, Fig. 6a). This injection parameters provided excellent results with regard to conformity of the electropherograms obtained by both injection methods as shown in Figs. 5 and 6. However, we achieved a ca. one thousand-fold improvement in sensitivity compared to hydrodynamic sample introduction for 1 s.

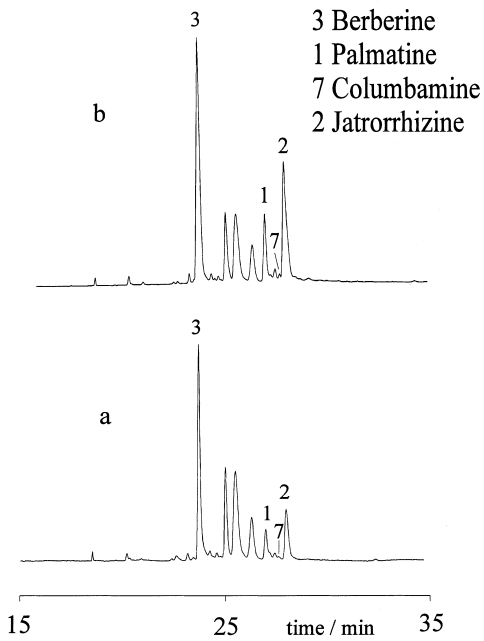

Fig. 5. Comparison of the normalised electropherograms from a MeOH extract of *B. vulgaris* obtained by hydrodynamic and electrokinetic injection. Conditions: (a) 1:1000 dilution of extract, electrokinetic injection with 16 kV and 8 s (+ to −) after preliminary pressure injection of 70% MeOH (v/v) for 1 s; (b) pressure injection for 1 s; running voltage 18 kV (+ to −), 15°C, UV detection at 240 nm; buffer: 200 m*M* ammonium acetate pH 3.1–MeOH (1:1, v/v); (for capillary dimensions and sample preparation see Sections 2.2 and 2.3).

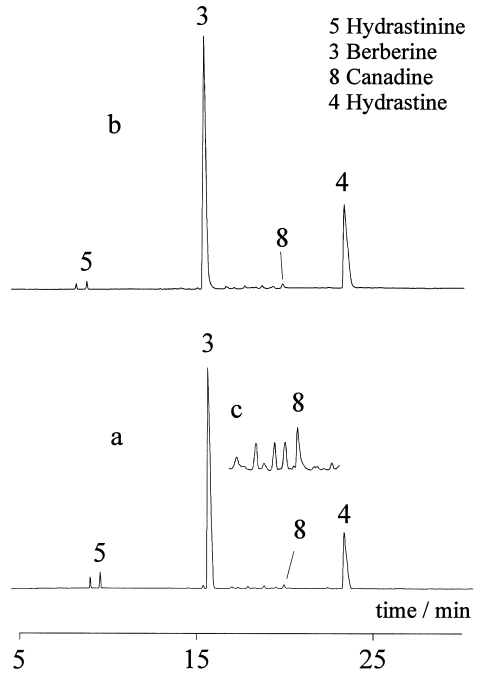

Fig. 6. Comparison of the normalised electropherograms from a MeOH extract of *H. canadensis* obtained by hydrodynamic and electrokinetic injection. Conditions: (a) 1:1000 dilution of extract, electrokinetic injection with 16 kV and 8 s (+ to −) after preliminary pressure injection of 70% MeOH (v/v) for 1 s; (b) pressure injection for one second; running voltage 20 kV (+ to −), 25°C, UV detection at 240 nm; buffer: 200 m*M* ammonium acetate pH 3.1–MeOH (1:1, v/v); (c) 1:100 dilution of extract, experimental conditions see (a); (for capillary dimensions and sample preparation see Sections 2.2 and 2.3).

The quaternary alkaloids palmatine (**1**), jatrorrhizine (**2**) or berberine (**3**) which are permanently charged in acid or even neutral solutions could be successfully analysed with FASI using MeOH for sample solution. The tertiary alkaloid hydrastine (**4**), however, gave rise to problems when dissolved in pure MeOH as a free base and injected through electromigration. No peak was observed when (**4**) was electrokinetically introduced into the capillary. In diluted MeOH the free base is protonated by water molecules and the substance could be electrokinetically loaded into the capillary without problems. As expected, a remarkable increase in sensitivity (about 5-fold) was obtained using a mixture of 10% acetic acid in 70% MeOH (v/v) (data not shown). This result is in agreement with data of Baeyens et al. [19] who found that electrokinetic injection of the alkaloid pilocarpine was improved when adding hydrochloric acid to the sample solution.

If further experiments for identification or structure elucidation of compounds (e.g., GC–MS) are necessary, the presence of acetic acid or inorganic ions would be a disadvantage. Thus 70% MeOH was used as solvent for preparing standard samples and after extraction of crude drugs the solutions were further diluted with 70% MeOH if analysed via FASI.

3.3. Comparison of hydrodynamic and electrokinetic injection

For conventional pressure injection in CE, the

amount of sample introduced can be influenced through the applied pressure and the injection time but is also dependent on the viscosity of the sample solution. Using electromigration methods, especially FASI, there are more facts to be considered. Although the introduced sample amount is mainly controlled by the applied voltage and the injection time, some additional aspects have to be taken into account: the conductance of the running buffer, the conductance of the sample solution and the electrophoretic mobilities of the analyte ions. A low conductance of the sample solution and the preinjected solvent plug, which provides a much higher field strength, also leads to an improved preconcentration of analytes through stacking. The higher the ionic strength of the electrolyte and the lower the ionic strength of the sample the higher is the improvement in concentration sensitivity.

In electrokinetic injection a bias results from different electrophoretic mobilities of substances [29]. Thus, the electropherograms obtained after hydrodynamic and electrokinetic injection are not directly comparable. Because the mobility of a compound is determined by the net charge and its molecular shape, representative electropherograms are only obtained if very similar substances being analysed. This is a disadvantage of electrokinetic injection. Since most of the samples contain structurally related compounds with only slight differences in size and charge, this effect is often negligible for qualitative analysis as clearly shown in Figs. 5 and 6.

In comparison with hydrodynamic injection (Fig. 5b), the ratio of signals for the berberis alkaloids palmatine (**1**), jatrorrhizine (**2**) and berberine (**3**) after FASI (Fig. 5a) remained almost constant. The peak height, however, decreases for the protoberberines in relation to three unidentified alkaloids. These might be bisbenzylisoquinolines for example berbamine (**9**) and oxyacanthine (**10**) eluting between 24 and 26 min. Because compounds (**9**) and (**10**) have higher electrophoretic mobilities than palmatine (**1**) or jatrorrhizine (**2**) their increased peak height compared to (**1**) and (**2**) is the consequence of an improved loadability. Obviously this is due to a double protonation of the bisbenzylisoquinolines (**9**) and (**10**).

In contrast to the poor reproducibility of corrected peak areas for berberine (**3**), the precision of migration times for FASI only slightly differed from that obtained through hydrodynamic injection (Table 1). Because the relative standard deviation of the measured peak heights was below 3% for FASI, this technique might be a useful tool for qualitative analysis. Due to the low reproducibility of determined peak areas the method is only applicable to quantitative analysis if an internal standard is used [19,25,30]. The limit of detection (LOD) for (**3**) was 1.3 ng ml^{-1} if the sample was injected electrokinetically. A similar value was achieved by Taylor et al. [18], who found that after electrokinetic injection of opiates the LOD was in the range of 10 ng ml^{-1}. A comparison of experimental data for both injection methods concerning precision and minimal detectable concentrations is given in Table 1.

3.4. Influence of structure on electrophoretic mobility

The extraordinarily high electrophoretic mobility of hydrastinine (**5**) (Fig. 6) cannot be explained sufficiently by its low-molecular-mass (M_r=207) compared to berberine (**3**) or hydrastine (**4**). If

Table 1
Comparison of hydrodynamic and electrokinetic injection for the determination of berberine

	Pressure injection (1 s)	Electrokinetic injection (16 kV, 8 s)
Corrected peak area (R.S.D., %)	5.0	12.1
Peak height (R.S.D., %)	2.5	2.2
Migration time (R.S.D., %)	1.4	1.6
LOD[a]	1.2 μg ml^{-1}	1.3 ng ml^{-1}

Running conditions: voltage: 20 kV; temperature: 25°C; wavelength: 240 nm; n=6.
[a] Limit of detection.

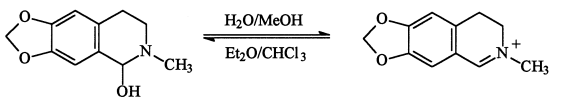

Fig. 7. Influence of solvent on the structure of hydrastinine (**5**).

analysed together with the biogenic amine tryptamine (**11**) having a molecular mass of 160, hydrastinine (**5**) eluted earlier (data not shown). For (**5**) this might be the consequence of the existence of two different structures depending on the nature of the applied solution. In polar solvents like water or MeOH the substance occurs as a quaternary base, whereas in nonpolar solvents like ether the molecule exists as a tertiary amine (Fig. 7) [2]. In fact the combination of the quaternary nitrogen and the low-molecular-mass of (**5**) results in a strongly reduced migration time. Thus elution order of hydrastis alkaloids can be clearly deduced from molecular mass and basic properties. Hydrastinine (**5**) having a low-molecular-mass and a strong basic character migrates first, followed by berberine (**3**) with a medium-molecular-mass and a strong basic character and finally hydrastine (**4**) with a medium-molecular-mass and medium basic properties. Canadine (**8**) normally occurs in *H. canadensis* in concentrations of ca. 1%. Its very low UV signal compared to berberine (**3**) or hydrastine (**4**) (Fig. 6a) is significantly improved at a higher sample concentration (Fig. 6c). Since the crude drug was not stored in a tightly closed container the low signal of (**8**) might be the result of an oxidation to berberine by exposure to air [31]. This oxidation is probably also a consequence of heating during soxhlet extraction. The different migration times of berberine (**3**) and canadine (=tetrahydroberberine) (**8**) can be easily explained by their basic properties because reduction of the quaternary alkaloid berberine (**3**) leads to the formation of the less basic tertiary amine canadine (**8**). For berberis alkaloids a comprehensive discussion concerning structure and electrophoretic mobility is given in [9].

4. Conclusions

The successful separation and identification of the major alkaloids of *Cortex Berberidis Radicis* and *Rhizoma hydrastis* was performed using a buffer solution consisting of MeOH–200 m*M* ammonium acetate pH 3.1 (1:1, v/v). In order to develop a selective and highly sensitive method for the determination of basic substances in crude plant extracts we used FASI for on-line preconcentration of highly diluted samples. After solid-phase extraction the method could also successfully applied to the CZE analysis of pyrrolizidine alkaloids present in *Tussilago farfara* L. in concentrations of only 1 ppm [32]. The coupling of CE with MS will provide a useful tool for the detection and identification of minor compounds especially when used in combination with electrokinetic injection. In addition, the application of CE–MS–MS will further enhance sensitivity and identification of unknown constituents present in crude mixtures for example plant extracts or biological fluids [33].

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