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Journal of Chromatography A, 717 (1995) 261–270

JOURNAL OF CHROMATOGRAPHY A

Analysis of the components of *Lycopus europaeus* L. in body fluids during metabolism studies Comparison of capillary electrophoresis and high-performance liquid chromatography

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Abstract

During pharmacokinetic studies with extracts obtained from medicinally used plants, analysis in body fluids is mainly performed by HPLC, an established separation method. In this paper high-performance capillary electrophoresis (HPCE) is investigated for its ability to separate such complex extracts. Crude extracts of *Lycopus europaeus* L. (Lamiaceae) are traditionally used against mild forms of hyperthyroidism. The metabolism of a 70% ethanolic extract with respect to some of its individual main components (rosmarinic and caffeic acid, luteolin-7-glucoside) and a mixture of the pure compounds were investigated using isolated perfused rat liver. After solid-phase extraction metabolites were determined using HPCE and HPLC separation techniques. A buffer solution composed of 0.05 mol l^{-1} Na_2HPO_4 at pH 7.0 with 30% acetonitrile was found to be the most suitable electrolyte for HPCE separation. The best mobile phase for isocratic HPLC was 0.03% TFA–acetonitrile (82:18, v/v). Data obtained with HPCE are in good accordance with those from HPLC; HPCE, however, is clearly more rapid and simple to perform.

1. Introduction

Plant extracts from *Lycopus europaeus* L. (Lamiaceae, "Wolfstrapp") are traditionally used in mild forms of hyperthyroidism. In animal experiments crude extracts show antithyreotropic and antigonadotropic effects [1–4]. Phenolic substances of the extract, e.g. rosmarinic and caffeic acid, are responsible for the biological effects [1–7]. The activity of the single phenolic constituents proved to be much smaller than that shown by the crude plant extracts. Therefore differences in metabolism between crude extracts and single compounds could account for such differences. To check this assumption the metabolism of an ethanolic extract of *Lycopus europaeus* L. was compared to that of three main components (rosmarinic and caffeic acid, luteolin-7-glucoside) and a mixture of these. For these investigations isolated perfused rat liver

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0021-9673/95/$09.50
SSDI 0021-9673(95)00492-0

was used as the model system [10,11]. Analysis of the obtained samples (perfusates) is usually performed by HPLC [8,9]. In this paper CE is investigated for its ability to separate such complex samples during metabolism studies.

2. Experimental

2.1. Apparatus and conditions

All CE analyses were carried out on a Waters Quanta 4000 capillary electrophoresis system (Waters, Eschborn, Germany) equipped with a UV detector set at 280 nm and an untreated fused-silica capillary (CS, Chromatographie-Service, Langerwehe, Germany) of 60 cm (total capillary length) × 75 μm I.D. and 360 μm O.D. The detection window was placed at 52 cm. Samples were introduced by hydrodynamic injection for 10, 12 or 20 s at the anodic end of the capillary. Running conditions were as follows: run time, 10 min; applied voltage, 25 kV (constant voltage, positive power supply); current, 273 μA; ambient temperature, 24–25°C. The electrolyte was a buffer consisting of 0.05 mol l^{-1} Na_2HPO_4 and 30% acetonitrile, adjusted to pH 7.0 with 1 *M* hydrochloric acid. The electrolyte was filtered through a 0.45-μm filter before use.

The HPLC system consisted of a pump (Waters M 45, Waters, Eschborn, Germany), a detector (Pye Unicam LC 3 UV, Cambridge, UK) set at 280 nm, an injector (Rheodyne 7161 + 7125) and an integrator (Pye Unicam CDP1, Cambridge, UK). A 10-μl injection volume and a column combination of a pre-column (LiChrosorb RP 18, 10 μm, 5 cm × 4 mm, Merck, Darmstadt, Germany) and an analytical column (LiChrosorb RP 18, 10 μm, 25 cm × 4 mm, Merck, Darmstadt, Germany) were used for all HPLC analyses. The mobile phase for isocratic HPLC separation was 0.03% TFA–acetonitrile (82:18, v/v) with a flow-rate of 1.2 ml min^{-1}.

Solid-phase extraction (SPE) was carried out on an Adsorbex SPU 19835 apparatus (Merck) with Bakerbond SPE Phenyl columns (3 ml size, Baker, Groß-Gerau, Germany). The column was conditioned using 3 ml methanol and 3 ml water. Sample preparation: 1 ml sample was acidified with 100 μl 1 *M* HCl. Sample application: 1–6 ml of acidified sample. Column washing: 1 × 3 ml water. Analyte elution: 2 × 3 ml methanol, evaporate to dryness, redissolved in 100–200 μl acidified methanol–water (1:1, v/v).

2.2. Model of the liver perfusion

According to the method described in Ref. [11] the isolated liver is connected to the perfusion apparatus described in Ref. [10]. For a single liver perfusion a period of 90 to 110 min is necessary. Samples (fractions 1–11) were taken at 10 min intervals. After an equilibration time of 10 min, the test substances were added to the perfusion medium (flow-rate 20 ml min^{-1}) over a 30-min interval (10–40 min). During each run an additional sample of perfusion medium was taken for chromatographic analysis shortly before liver passage (VL) usually at 30 min in order to determine the real concentration of the test compounds. This is necessary as loss of substance occurs within the system. The difference between the concentration before (determined from VL) and after liver passage (determined from F1–F11) is used to calculate the metabolic rate of the substances after the following scheme [12]:

–Before liver passage: $VL \cdot 20 \cdot 30 = x$ (μg) = 100%.

–After liver passage: $(F1–F11) \cdot 20 \cdot 10 = y$ (μg).

–Metabolic rate (%): $100 - \frac{100 \cdot y}{x}$.

2.3. Materials

Perfusion medium for the in vitro model

As perfusion medium a modified Krebs–Henseleit solution was used: NaCl 0.119 mol l^{-1}, KCl $4.74 \cdot 10^{-3}$ mol l^{-1}, $NaHCO_3$ $2.5 \cdot 10^{-2}$ mol l^{-1}, KH_2PO_4 $1.16 \cdot 10^{-3}$ mol l^{-1}, $MgSO_4$ $1.16 \cdot 10^{-3}$ mol l^{-1}, $CaCl_2$ $2.0 \cdot 10^{-3}$ mol l^{-1}, MOPS (3-N-morpholinopropanesulfonic acid)

$3.0 \cdot 10^{-3}$ mol l^{-1}, glucose $1.15 \cdot 10^{-2}$ mol l^{-1}, bovine serum albumin (BSA) 0.5% and sodium taurocholate $2 \cdot 10^{-5}$ mol l^{-1}. BSA was of chemical grade and purchased from UCB (Brussels, Belgium). All other chemicals used for the perfusion medium were of analytical grade and purchased from Merck or Sigma (Munich, Germany).

Acetonitrile for CE and HPLC was of ultragradient quality and obtained from Baker. Na_2HPO_4 and methanol for the buffer solution were obtained from Merck. Natural substances (caffeic and rosmarinic acid, luteolin, luteolin-7-glucoside and protocatechualdehyde) were purchased from Roth (Karlsruhe, Germany).

Preparation of the test substances

An amount of 200 ml of the 70% ethanolic extract of *Lycopus europaeus* L. (obtained from Finzelberg, Kleinostheim, Germany) was evaporated until the amount of ethanol was less than 1% (data obtained from headspace GC). The residue was redissolved in 50 ml perfusion medium and filtered through an 8-μm filter. Caffeic acid ($5.5 \cdot 10^{-4}$ mol l^{-1}) was dissolved in 100–200 μl hot water and diluted with physiological buffer. Luteolin-7-glucoside is only poorly soluble in the perfusion medium. A complete solution can be obtained by dissolving $1.1 \cdot 10^{-4}$ mol l^{-1} luteolin-7-glucoside in 30–50 μl 2 *M* NaOH with ultrasound treatment. This solution was filled up with buffer to 50 ml. Rosmarinic acid is readily soluble in the buffer.

2.4. HPLC of perfusates

HPLC of standard mixtures of the three main components

For HPLC analysis of the perfusates from liver perfusion, samples were deproteinized with 3% trichloroacetic acid and centrifuged at 4000 *g*, the supernatant was used for HPLC. Metabolites in the perfusates were determined after solid-phase extraction on phenyl columns.

Recovery

A mixture of three main components of *Lycopus europaeus* L. (for structures see Fig. 1) was dissolved in physiological buffer, acidified with 100 μl 1 *M* HCl per ml, injected directly onto the HPLC system and detected at 280 nm. The peak areas were taken to 100%. The sam-

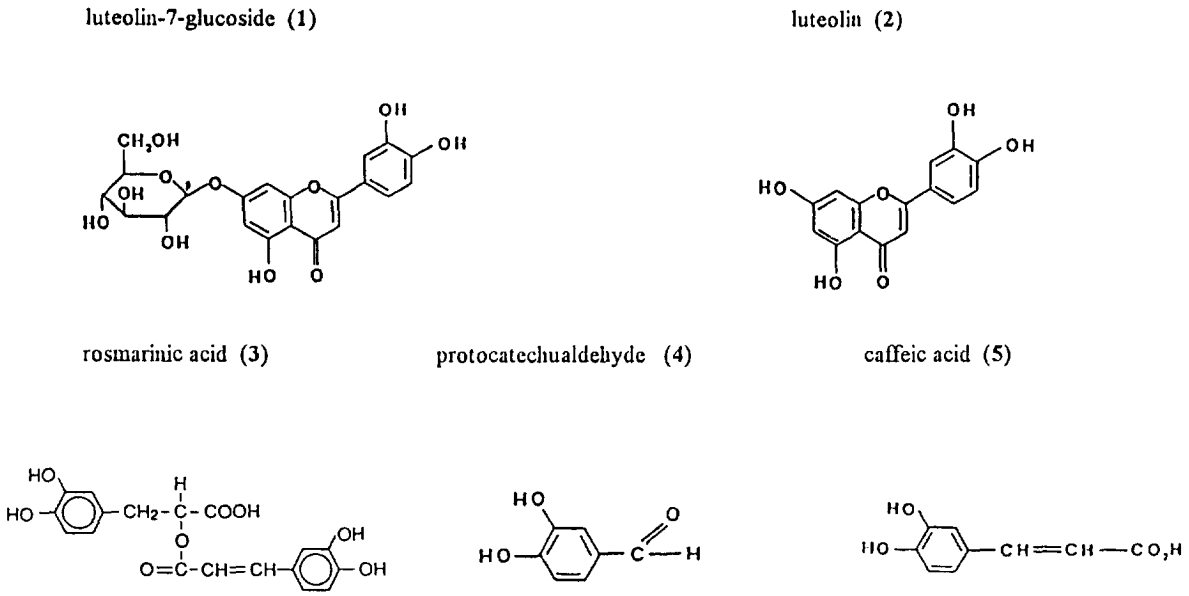

Fig. 1. Structures of five substances from the ethanolic extract of *Lycopus europaeus* L.

Table 1
Recovery rates for three main components after SPE

Component	Concentration (mol l^{-1})	Recovery rate (%)
Luteolin-7-glucoside	$2.45 \cdot 10^{-3}$	82 ± 6.6
Caffeic acid	$7.20 \cdot 10^{-4}$	95 ± 8.8
Rosmarinic acid	$2.78 \cdot 10^{-3}$	94 ± 7.3

The concentration of the substances corresponds to the concentration found in the crude extract.

ples were acidified and concentrated via solid-phase extraction (SPE, $n = 5$).

3. Results and discussion

3.1. Recovery

For recovery experiments a comparison was made between the direct injection of the three main components into the HPLC system followed by injection of samples which had been purified by solid-phase extraction (SPE). Table 1 shows recovery rates between 82 and 95% when the three compounds were used in a concentration as obtained for the crude plant extract. Table 2 shows that a variation between ca. 75 up to 105% of the recovery exists dependent on the amount of each individual compound over a range from 0.01 to 1 mg ml^{-1}.

Recovery (± R.S.D.) over a concentration range from 0.01–1 mg ml^{-1} ($n = 3$) varies as shown in Table 2.

3.2. Efficiency

Fig. 2 shows the CE and HPLC separations of the crude extract; the separation of a mixture of the five components of *L. europaeus* is depicted in Fig. 3. All five components can be detected within 6 min following CE analysis, whereas only four components can be found after 30 min using HPLC isocratic elution. CE analysis leads to better peak shape and efficiency and, moreover, a better resolution between peak 4 and 5 is obtained. In Table 3 efficiency, expressed in terms of number of theoretical plates (N), is compared for CE and HPLC analysis for the crude extract. Table 3 clearly shows that CE provides a higher separation efficiency in comparison to HPLC.

3.3. Linearity

To determine the linearity for both techniques plots of peak area versus concentration over the range 0–1060 μg ml^{-1} were made for a mixture of the three main components of *L. europaeus*. CE and HPLC regression equations of the curves and correlation coefficients as presented in Table 4 are in good accordance.

3.4. LOD (limit of detection)

In Table 5 the limits of detection for CE and HPLC analysis for the three main components are compared using the signal-to-noise ratio (2:1) at 280 nm. Data are based on three replicate injections with 20 s HD injection for CE and 10 μl injection for HPLC. HPLC proved

Table 2
Recovery rates (%) for three components after SPE over a range from 1.0–0.01 mg ml^{-1}

Substance	Concentration (mg ml^{-1})				
	1.0	0.25	0.1	0.025	0.010
Luteolin-7-glucoside	82 ± 6.2	87 ± 5.0	87 ± 4.2	105 ± 5.7	107 ± 8.7
Caffeic acid	103 ± 1.2	87 ± 5.9	88 ± 6.6	87 ± 8.3	106 ± 8.7
Rosmarinic acid	92 ± 4.4	83 ± 4.0	76 ± 1.0	87 ± 7.8	91 ± 8.6

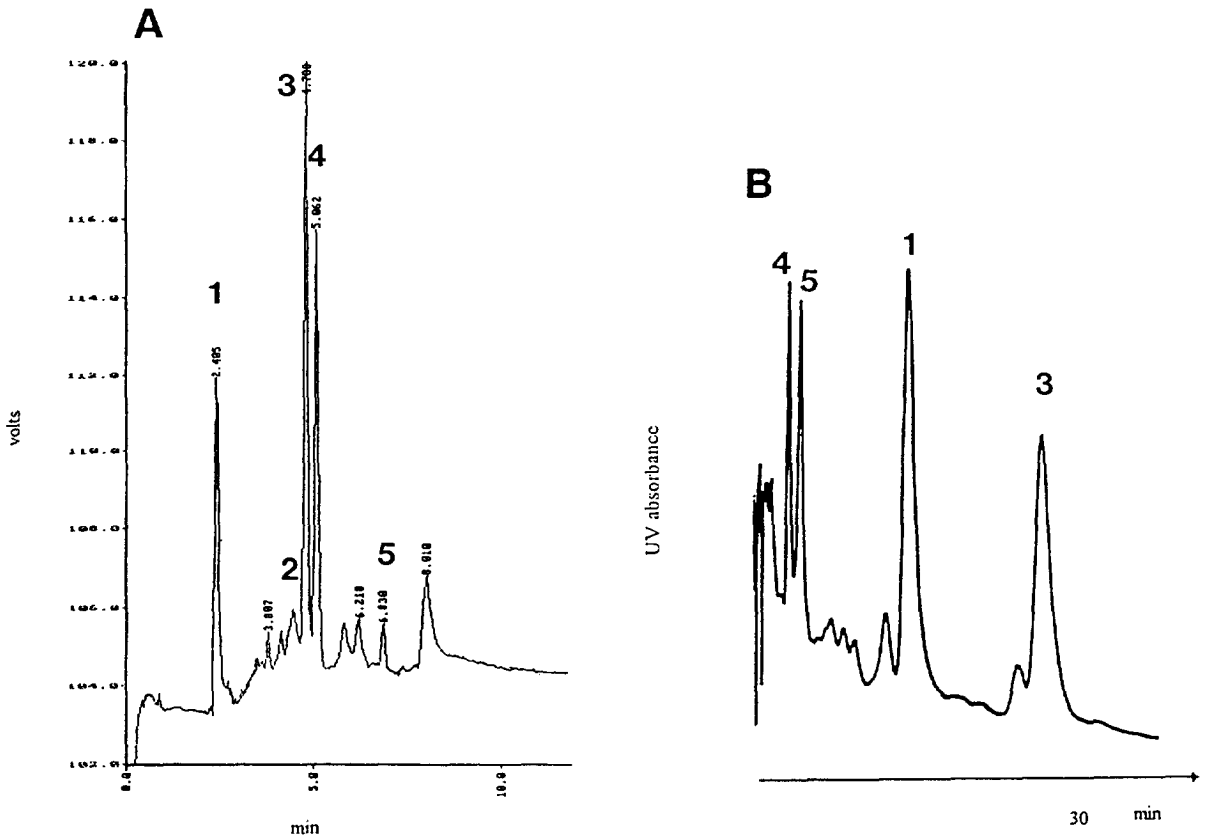

Fig. 2. Separation of the crude extract with both techniques. Peaks: 1 = luteolin-7-glucoside, 2 = luteolin, 3 = rosmarinic acid, 4 = protocatechualdehyde, 5 = caffeic acid. (A) CE analysis. Conditions: sample, original 70% ethanolic extract dissolved with water (1:1, v/v), filtered through 0.45-μm before use; buffer, 0.05 mol l^{-1} Na_2HPO_4, pH 7.0, with 30% acetonitrile; capillary, 60 cm (52 cm to detector) × 75 μm I.D.; voltage, 25 kV; current, 273 μA; temperature, 24–25°C; detector, UV 280 nm; injection, 12 s hydrodynamic. (B) HPLC analysis. Conditions: sample, see above; column combination: LiChrosorb RP 18, 10 μm, 5 cm × 4 mm and LiChrosorb RP 18, 10 μm, 25 cm × 4 mm; eluent, 0.03% trifluoroacetic acid–acetonitrile (82:18, v/v); flow-rate, 1.2 ml min^{-1}; temperature, 25°C; detector, UV 280 nm; injection, 10 μl.

to be more sensitive for luteolin-7-glucoside and caffeic acid. This is explained by the small size of the detector flow cell used with CE.

3.5. Precision

To control the reproducibility of peak areas for both techniques, ten injections of a mixture of caffeic acid ($7.2 \cdot 10^{-4}$ mol l^{-1}), rosmarinic acid ($2.8 \cdot 10^{-3}$ mol l^{-1}) and luteolin-7-glucoside ($2.45 \cdot 10^{-3}$ mol l^{-1}) were made. All substances were dissolved in buffer and concentrated via SPE before use. The relative standard deviation (R.S.D.) in peak area is shown in Table 6. Differences in R.S.D. between both methods can be explained by the different injection volumes used (CE e.g. 80 nl, HPLC e.g. 10 μl).

3.6. Metabolism studies

Metabolic rates

Metabolism studies were carried out using the model of the isolated perfused rat liver [10,11] as described in the Experimental section. After an equilibration time (10 min), the test substances (crude extract, individual components, mixture

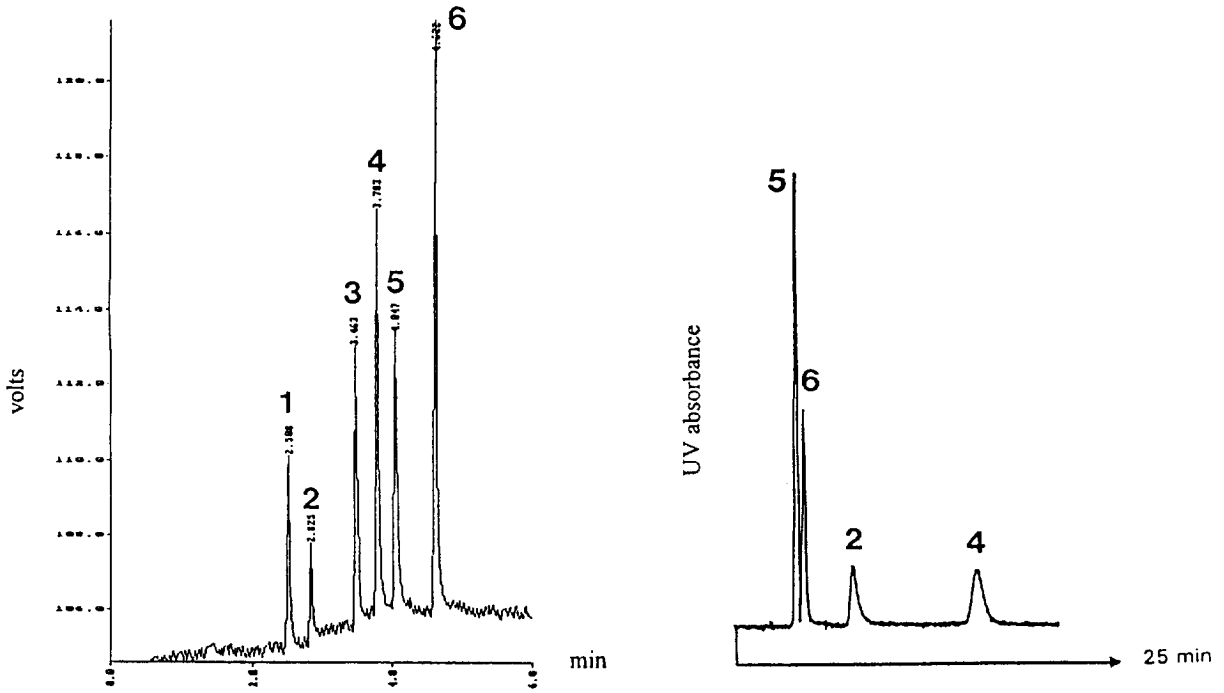

Fig. 3. Separation of a standard mixture with both techniques. Peaks: 1 = methanol, 2 = luteolin-7-glucoside, 3 = luteolin, 4 = rosmarinic acid, 5 = protocatechualdehyde, 6 = caffeic acid. (Left) CE analysis. Injection, 20 s hydrodynamic; for conditions see Fig. 2. (Right) HPLC analysis. For conditions see Fig. 2.

Table 3
Efficiency for both techniques

Substance	N (CE)	N (HPLC)
Luteolin-7-glucoside	$3.0 \cdot 10^3$	$1.3 \cdot 10^2$
Luteolin	$1.6 \cdot 10^2$	n.d.
Caffeic acid	$2.3 \cdot 10^4$	$8.7 \cdot 10^1$
Rosmarinic acid	$2.9 \cdot 10^4$	$2.9 \cdot 10^2$
Protocatechualdehyde	$8.9 \cdot 10^4$	$4.2 \cdot 10^1$

Table 4
Linearity for both techniques

	CE	HPLC
Luteolin-7-glucoside	$y = -0.041x + 0.049$ ($r = 0.9993$)	$y = -0.669x + 0.284$ ($r = 0.9994$)
Caffeic acid	$y = -0.552x + 0.321$ ($r = 0.9998$)	$y = 1.697x + 0.195$ ($r = 0.9985$)
Rosmarinic acid	$y = -0.575x + 0.108$ ($r = 0.9987$)	$y = 0.490x + 0.103$ ($r = 0.9993$)

Table 5
Limit of detection

Substance	Concentration detection limit (mol l^{-1})	
	CE	HPLC
Luteolin-7-glucoside	$5.6 \cdot 10^{-5}$	$2.2 \cdot 10^{-5}$
Caffeic acid	$5.5 \cdot 10^{-5}$	$5.5 \cdot 10^{-6}$
Rosmarinic acid	$2.8 \cdot 10^{-5}$	$2.8 \cdot 10^{-5}$

Table 6
Standard deviation of peak areas obtained by CE and HPLC

	CE	HPLC
Luteolin-7-glucoside	5.6%	3.9%
Caffeic acid	8.1%	4.1%
Rosmarinic acid	9.8%	4.0%

of the components) were added between 10 and 40 min to the flowing medium over a period of 30 min. The perfusion medium during liver perfusion was collected in 10 min intervals (= fractions F1–F11). For determining metabolism rates the difference between the concentration before (VL, measured once at 30 min) and after liver passage (sum of fractions) was calculated (see Experimental).

Standard mixtures and individual components were administered at the same concentration as in the crude extract. Using the analytical procedure developed above, first results ($n = 3$) were obtained with the three main components.

The results of liver perfusion of the three main components from the crude extract are shown in Fig. 4. Concentrations of the components in the different fractions were measured with both CE and HPLC, after purification of the samples by

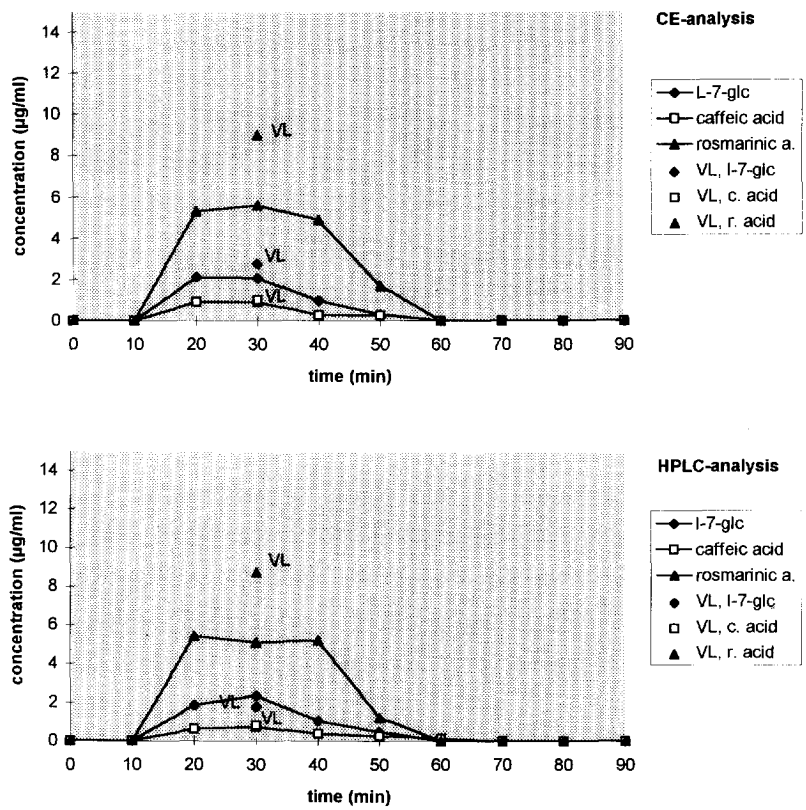

Fig. 4. Concentration of three components of the crude extract in perfusion medium before (VL) and after liver passage as determined by CE analysis (top) and HPLC analysis (bottom); addition of $2.2 \cdot 10^{-3}$ mol l^{-1} of luteolin-7-glucoside (l-7-glc), $2.78 \cdot 10^{-3}$ mol l^{-1} rosmarinic acid (r. acid) and $5.5 \cdot 10^{-4}$ mol l^{-1} caffeic acid (c. acid) per min over 30 min.

SPE. When the three substances are applied in a mixture, similar curves can be obtained; but the decline in concentration of luteolin-7-glucoside is more pronounced now (see Fig. 5).

After application of each of the three components individually the curves presented in Figs. 6a–c were observed. Each figure shows the analysis with both separation techniques.

Differences in the degree of metabolization between the applied preparations are clearly visible. Metabolic rates calculated from Figs. 4–6 are shown in Fig. 7 for CE and in Fig. 8 for HPLC. The results obtained by both methods are in principle comparable. Rosmarinic acid shows a larger deviation as this is the most sensitive compound against oxidative influences. Interestingly, the metabolic rates differ between the different forms of preparation. Although luteolin-7-glucoside is preferentially metabolized, the rate of its metabolism differs greatly, from 30–35% when administered in the crude extract, over 55–60% when applied as an individual compound, to 65–70% when given in the standard mixture of the three pure compounds. Caffeic acid is clearly better metabolized when given alone; rosmarinic acid, on the other hand, seems to be better metabolized from the crude extract or from the standard mixture. Although these data are preliminary, they indicate that metabolic rates of individual compounds are influenced by accompanying substances; this points to interactions in the metabolism of secondary constituents when applied in crude plant extracts versus application as a pure

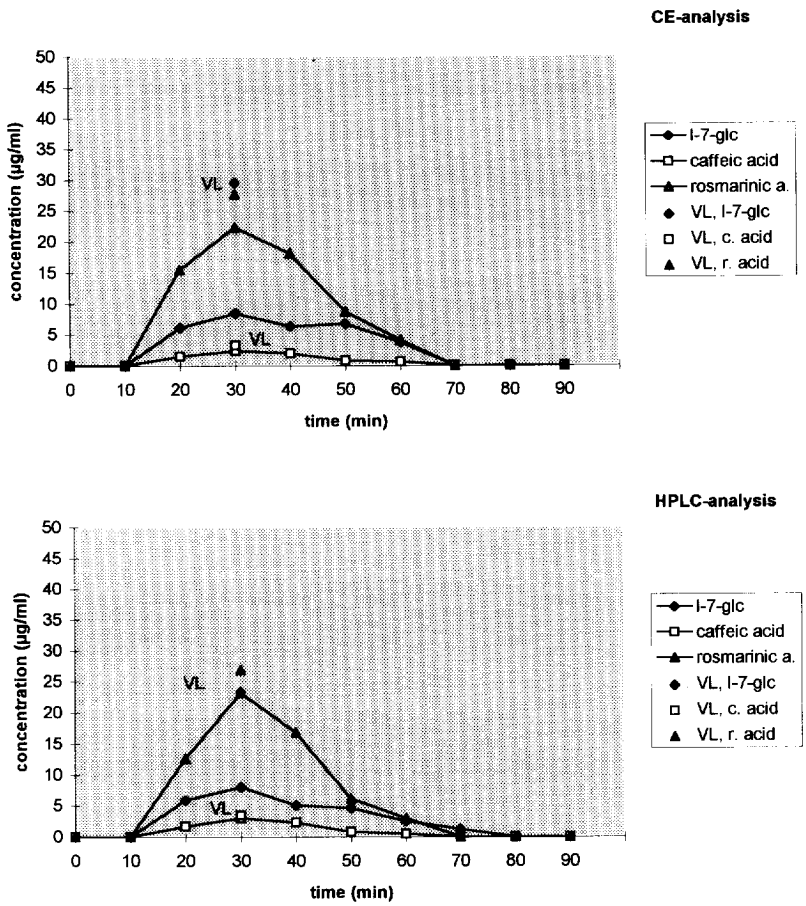

Fig. 5. Concentration of three components applied as a mixture before and after liver passage. Concentrations are the same as in the crude extract (Fig. 4). (top) CE analysis, (bottom) HPLC analysis.

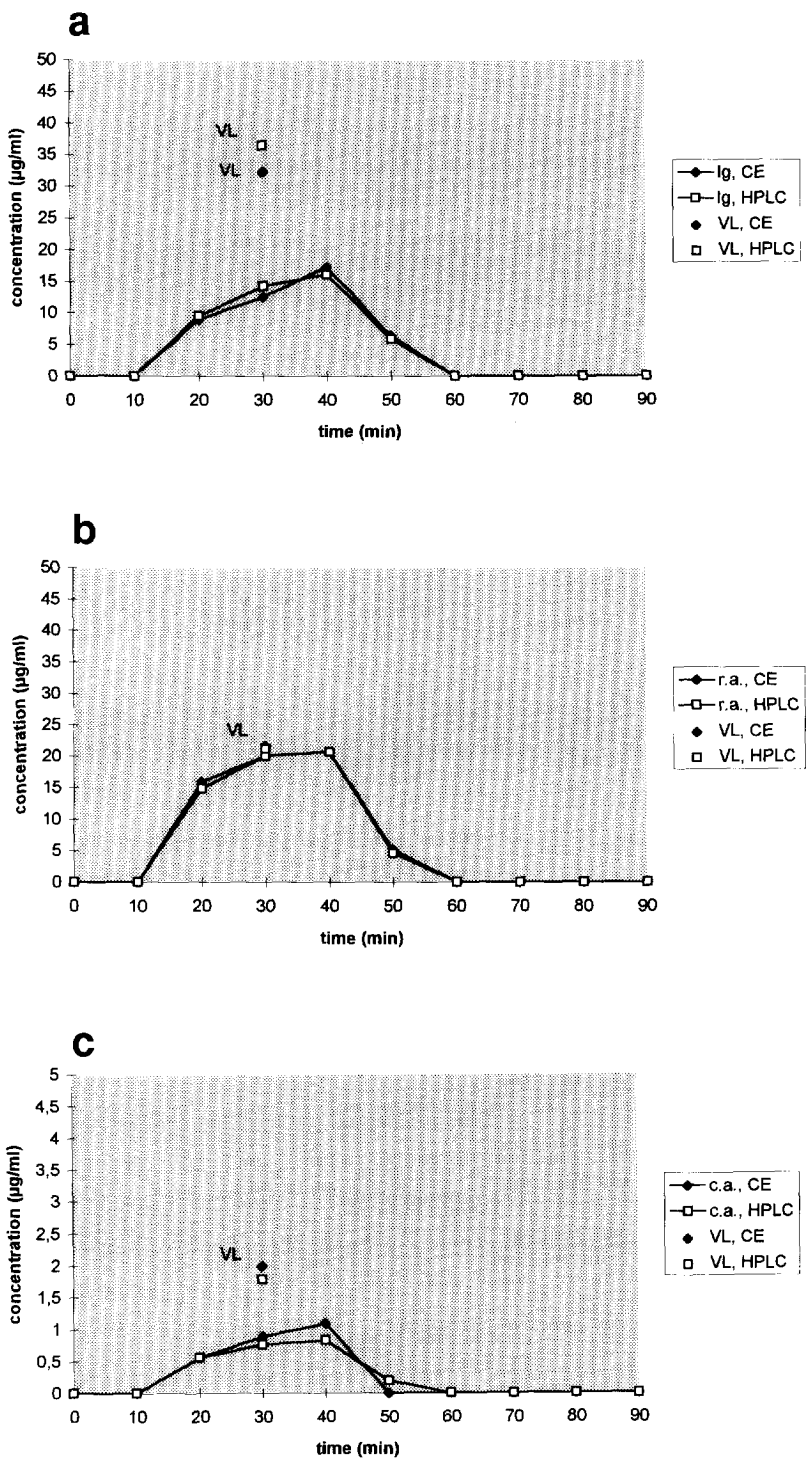

Fig. 6. Concentrations of the components before and after liver passage after individual application of $2.2 \cdot 10^{-3}$ mol l^{-1} luteolin-7-glucoside (a), $2.78 \cdot 10^{-3}$ mol l^{-1} rosmarinic acid (b) and $5.5 \cdot 10^{-4}$ mol l^{-1} caffeic acid (c) per min over 30 min.

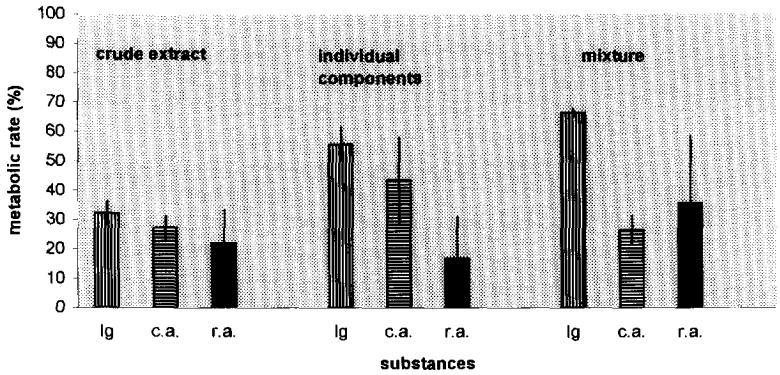

Fig. 7. Comparison of metabolic rates (determination by CE; $n = 3$).

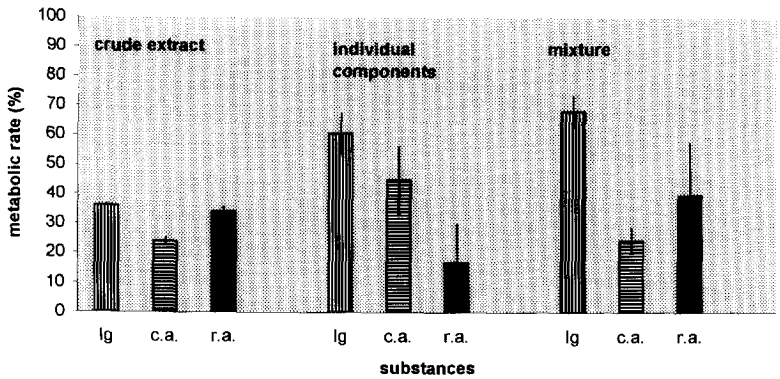

Fig. 8. Comparison of metabolic rates (determination by HPLC; $n = 3$).

compound. These data, of course, need further substantiation.

In conclusion, CE determination of the compounds in question exceeds HPLC in that it is quicker, less expensive and provides a better resolution. Crude plant extracts as well as crude liver perfusates can be well separated after a short purification. CE is a usable tool to elucidate the metabolism of secondary plant constituents.

References

[1] H. Winterhoff, H.G. Gumbinger and H. Sourgens, Planta Med., 54 (1988) 101–106.
[3] H. Winterhoff, H.G. Gumbinger, U. Vahlensieck, F.H. Kemper, H. Schmitz and B. Behnke, Arzneim. Forsch./Drug Res., 44 (1994) 41–45.
[4] F.H. Kemper, H. Winterhoff, H. Sourgens and K.D. Niehaus, Planta Med., 33 (1978) 311.
[5] G. Madaus, Fr.E. Koch and G. Albus, Z. Gesamte Exp. Med., 109 (1941) 411–424.
[6] B. Wylde, A. Sosa, F. Winternitz, H. Winterhoff and F.H. Kemper, Planta Med., 39 (1980) III-62.
[7] H.G. Gumbinger and H. Winterhoff, Z. Phytother., 8 (1987) 172–174.
[8] H.G. Gumbinger, U. Vahlensieck and H. Winterhoff, Planta Med., 59 (1993) 485–578.
[9] D. Strack and H.G. Gumbinger, Personal communication.
[10] C. Egen-Schwind, R. Eckard and F.H. Kemper, Planta Med., 58 (1992) 301–388.
[11] T. Sugano, K. Suda, N. Shimada and N. Oshino, J. Biochem., 83 (1978) 995–1007.
[12] H. Wojciechowski, unpublished results.