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A new method of capillary electrophoresis for metabolites of coumarin

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The metabolism of coumarin has been very widely investigated compared with other natural compounds. We know today that genetic variations lead to human groups with a high or low coumarin-metabolism. An effective capillary-electrophoresis method has been developed to study these effects on metabolic patterns. The seven most important metabolites can be analysed in one step. Therefore also small volume samples can be examined for phase-I-reactions (hydroxylation of coumarin) as well as for secondary reactions (e.g. glucuronidation or decomposition of the lactone-structure).

1. Introduction

The widespread plant constituent coumarin (benzo-1,2-pyrone) is an active ingredient of extracts, such as *Meliloti herba*/Sweet clover and also a synthetic compound. It is successful for the therapy of chronic-venous-insufficiency. Every person also takes in coumarin in low concentrations as part of fruits and vegetables in their daily nutrition. The metabolism of benzo-1,2-pyrone has been investigated extensively, in contrast to most plant constituents. Molecular-biological studies demonstrate the important role of specific cytochrome P450-isozymes occuring principally in the liver. Coumarin is metabolized by CYP2A-6 (humans) and CYP2A-5 (mice). Although both mainly hxdroxylate coumarin to 7-hydroxycoumarin (umbelliferone), 3-hydroxycoumarin is the most important metabolite in the rat (Table 1). In addition, further hydroxylations of minor importance occur at various positions on the ring system [1–2]. These results indicate the differences in coumarin-metabolism depending on species. In humans, variations of the gene responsible for enzymatic configuration result in patients with different metabolizing rates ("high- or low-metabolizers") [3]. Further investigations are necessary to understand if these preclinical effects can be observed in patients. A valid analytical technique is needed to determine metabolites in body fluids such as blood or urine, since knowledge of the metabolic pattern is necessary to estimate the consequences of genetic variation.

Recently, methods were published which demonstrate the successful analysis of simple and complex (mono-)saccharides and a valid chiral separation of enantiomers with capillary electrophoresis [4–7]. In theory, the separation of coumarin-metabolites by capillary electrophoresis should therefore also be possible. Experience in the analysis of enantiomers is necessary for the separation of the phase-I-metabolites, which differ only slightly in chemical structure. Coumarin is metabolised in phase I to hydroxycoumarins which are glucuronidated in phase II, so that here information available for saccharide-analyses can be exploited. Based on previous methods published for analyzing selected coumarins by capillary electrophoresis [8–9], a method is presented in this paper which quantifies all pharmacologically important metabolites of coumarin in one analytic step. The current questions about the (clinical) pharmacology of coumarin can be investigated with an ecological-compatible analytical technique, because it is free of organic liquids, in contrast to the usual chromatographic methods.

2. Investigations, results and discussion

2.1. Influence of pH

A higher pH-value increases the fraction of negatively-charged silanol-groups in the capillary and also the electroosmotic flow (EOF). The higher pH-value also influences the dissociation and hence the electric charge of the substance being analysed. Although cations and neutral molecules migrate faster, the migration of the anions is lower. The best separation is theoretically obtained if the pH is placed between the pKa-values of the analytes [10].

The selection of the right pH-value is difficult in metabolism-studies, because the different pathways of metabolism lead to a wide range of pKa-values depending on the chemical structure of the metabolite. For this study, a pH-value of 10.0 has been selected, which reflects the chemical structure of the coumarin-molecule and the hydroxyl groups of the phase-I-metabolites. In general, the pH-value influences the migration times of all coumarin-metabolites. With increasing pH-values the migration times lengthen. If a lower pH-value than 10.0 is chosen, a qualitative separation is not possible.

2.2. Influence of electrolyte concentration

An electrolyte-concentration between 10 and 50 mmol is recommended for the CE-equipment used. Thereby the pherogram gives excellent and symmetric peaks. The concentration of all ions (buffer and sample) has to be constant, so that the ions of the samples do not influence the electric field. Certainly a too high ion-concentration is dangerous, because effects of electrolysis can appear, which prevent a succesful analysis.

For this study a electrolyte-concentration of 25 mM has been selected. The ions of the samples do not disturb the capillary electrophoresis, because the theoretical complete ion concentration of coumarin metabolites is only in the range of μmols. During the validation of the analytical proceeding, lower (10 mM) and higher (30 mM) concentrations of the buffer were tested, which lead to more disadvantageous separation-profiles.

2.3. Influence of organic liquids

The mobility of an ion depends on the viscosity according the equation of electric mobility:

$$\mu_e = 1/6 \times \pi^{-1} \times q \times \eta^{-1} \times r^{-1}$$

[μ_e: electric mobility; q: electric charge; η: viscosity; r: radius of the ion].

The addition of an organic liquid to the buffer diminishes the viscosity and the dielectricity constant the mobility. Because methanol has a positive solvation effect on coumarins in aqueous solutions, this liquid could be used as an additive to the buffer. A comparison of a separation without methanol and with the organic liquid demonstrates the advantage of the additive, because the separation is now exacter.

2.4. Influence of temperature

The temperature influences the viscosity of the buffer and the electroosmotic flow (EOF). In general, the electric conductance increases at higher temperatures, whereas the migration times are diminished.
To find the optimal temperature for the separation of the coumarin metabolites, analyses were carried out at 20 °C, 30 °C, 40 °C, 50 °C and 60 °C. The low temperature of 20 °C was selected for further investigations, because this temperature guarantees a separation of the peaks including the base lines.

2.5. Quality of the method

In the Fig., a typical pherogram is shown. The metabolites appear between 3 and 12 min in a short period. To demonstrate the quality of the new method, repeated tests ($n = 6$) were carried out. Table 2 demonstrates the migration times (t_m) and their reproducity, and Table 3 gives the resolutions R_S, retention-factors k and selectivity-factors α of the coumarin-metabolites. For simplification, only those results found at one wavelength ($\lambda = 210$ nm) were interpreted here. This restriction has also the advantage that the method described can be used for capillary-electrophoresis-hardware with a standard UV-detector. Linearity is given for all metabolites in concentrations between 2 and 1000 mg/l, and the detection limit lies at 1 mg/l, which corresponds to molar concentrations from 6.85×10^{-6} to 1.41×10^{-5} M. The reproducibility is demonstrated by the low values of the relative standard deviation (1.34–3.84%). Coumarin was the marker substance to calculate retention and selectivity.
The method described allows the succesful investigation of the most important coumarin metabolites in one step without derivatisation. Difficult preparation of the samples at the beginning of the analysis can therefore be avoided. Additionally, the possibility of errors is reduced by the simple technique. A comparison with HPLC-methods shows that the CE-method is acceptable for its quality and precision. In addition, capillary electrophoresis has important ecological advantages.

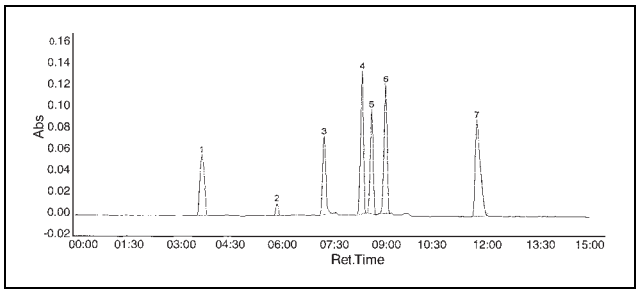
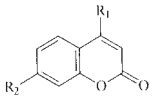


Fig.: Separation of coumarin-metabolites by capillary electrophoresis. Abbreviation of the peaks: 1: coumarin; 2: 7-hydroxycoumaringlucuronide; 3: 6,7-dihydroxycoumarin; 4: 7-hydroxycoumarin; 5: 3-hydroxycoumarin; 6: 4-hydroxycoumarin; 7: 3,7-dihydroxycoumarin. Conditions: buffer 0.025 mM $Na_2B_4O_7$/NaOH pH 10.0 in H_2O/MeOH (9 + 1); temperature: 20 °C; voltage: 30 kV; wavelength for detection: 210 nm; further information is given in the text

Table 1: Main coumarin metabolites in rats, humans and mice



Substance	R_1	R_2	Species
Coumarin	H	H	
3-Hydroxycoumarin	OH	H	Rat
Umbelliferone	H	OH	Human, Mouse

Table 2: Reproducibility of the migration times t_m with SD and RSD of coumarin-metabolites ($n = 6$)

Metabolite	t_m (min)	SD	RSD (%)
Coumarin	3.39	0.05	1.44
7-Hydroxycoumaringlucuronide	5.59	0.21	3.84
6,7-Dihydroxycoumarin	7.15	0.10	1.34
7-Hydroxycoumarin	8.21	0.12	1.46
3-Hydroxycoumarin	8.37	0.13	1.51
4-Hydroxycoumarin	8.81	0.32	3.63
3,7-Dihydroxycoumarin	11.45	0.31	2.75

Table 3: Resolutions R_s, retention-factors k and selectivity-factors α of coumarin-metabolites

Metabolite	R_S	k	α
Coumarin	11.64		
7-Hydroxycoumaringlucuronide	9.51	4.59	1.339
6,7-Dihydroxycoumarin	6.34	6.15	1.172
7-Hydroxycoumarin	1.75	7.21	1.023
3-Hydroxycoumarin	1.75	7.37	1.059
4-Hydroxycoumarin	2.39	7.81	1.339
3,7-Dihydroxycoumarin	15.58	10.45	

3. Experimental

3.1. Chemicals

Coumarin, esculetin and umbelliferone were purchased from Sigma (Deisenhofen, Germany). 3,7-Dihydroxycoumarin and 3-hydroxycoumarin were synthesized according to the literature [11–12]. The preparation of 7-hydroxycoumarin glucuronide was described earlier [9]. All other chemicals came from E. Merck (Darmstadt, Germany). The deionized water was double-distilled. All reagents were of analytical grade.

3.2. Equipment

The UNICAM-hardware included a crystal capillary-electrophoresis-system model 310 with a crystal 240 diode array detector. The fused silica capillaries, which were obtained from Supelco (Bellefonte, USA), had a length between injection and detector of 0.488 m with a I.D. of 75 μm. The UNICAM-software (Vers. 4880) controlled the separation and integrated the data.

3.3. Method

Before and between tests the capillary was conditioned for 3 min with 1 N sodium hydroxide and additionally for 2 min with a borate-buffer at a voltage of 30 kV. An aqueous-methanolic solution (9 parts water + 1 part methanol) of 0.025 M sodium tetraborat was adjusted on pH 10.0 with 1 N sodium hydroxide as buffer. Further conditions were a voltage of 30 kV (ramp 6 kV/s) and a temperature of 20 °C during the separations. During the validation of the method, different parameters were used: buffer without methanol; buffer-concentration: 0.010 M, 0.025 M or 0.030 M; pH-value: 9.9, 10.0 or 10.1; temperature: 20 °C, 30 °C, 40 °C, 50 °C or 60 °C. After dissolving in the buffer the substances were injected hydrodynamically (50 mbar/10 s, corresponding to 39 nl) and detected in a wavelength-range from 190 nm to 360 nm.

References

1 Pelkonen, O.; Raunio, H.; Rautio, A.; Mäenpää, J.; Lang, M., A.: J. of the Irish Colleges of Physicians and Surgeons **22**, Suppl. 24 (1993)
2 Tegtmeier, M.; Legrum, W.: Arch. Pharm. **327**, 299 (1994)
3 Fernandez-Salquero, P.; Hoffman, S. M. G.; Chollerton, S.: Amer. J. Human Genet. **57**, 651 (1995)
4 Kuhr, W. G.: Anal. Chem. **62**, 403 (1990)
5 Engelhardt, H.; Beck, W.; Kohr, J.; Schmitt, T.: Angew. Chem. **105**, 659 (1993)
6 Noe, C. R.; Freissmuth, J.; Rothley, D.; Lachmann, B.; Richter, P.: Pharmazie **51**, 868 (1996)
7 Schmid, M. G.; Wirnsberger, K.; Gübitz, G.: Pharmazie **51**, 852 (1996)
8 Bogan, D. P.; Deasy, B., O'Kennedy, R.; Smyth, M. R.; Fuhr, U.: J. Chromatograpy B **663**, 371 (1995)
9 Bogan, D. P.; Thornes, R. D.; Tegtmeier, M.; Schafer, E. A.; O'Kennedy, R.: Analyst **121**, 243 (1996)
10 Mizukami, M.; Hanai, T.: J. High Res. Chromatogr. **14**, 561 (1991)
11 Shaw, K.; McMillan, A.: J. Org. Chem. **21**, 601 (1956)
12 Billek, G.: Monatsh. Chem. **92**, 343 (1961)

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