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Application of micellar electrokinetic capillary chromatography to the determination of flavonoid drugs

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ABSTRACT

The use of micellar electrokinetic capillary chromatography is described for the determination of flavonol-3-O-glycosides. A 72-cm fused-silica capillary column and a 50 m*M* sodium dodecyl sulphate–20 m*M* sodium borate running buffer (pH 8.3) were used for the electrophoretic separations. The samples were injected onto the capillary column in the range 10–1000 pg. Results are given for quercetin-, kaempferol- and isorhamnetin-3-O-glycosides and for a standardized *Ginkgo biloba* extract. The results are compared with those obtained by reversed-phase high-performance liquid chromatography.

INTRODUCTION

Capillary electrophoresis (CE) [1] is becoming increasingly recognized as an important analytical separation technique as a result of its speed, reproducibility, accuracy and potential for automation. In addition, CE does not require any staining procedure, and the sample bands subsequent to separation are detected by on-line ultraviolet (UV) detection in the same way as for high-performance liquid chromatography (HPLC). From a review of the literature it is clear that a large variety of compounds, including amino acids [2], peptides [3], proteins [4], oligonucleotides [5], DNA fragments [6], vitamins and drugs [7] have been separated and analysed by CE. However, this technique has not yet been applied to the separation of flavonoids. These compounds are widely distributed plant metabolites, with structures based on 2-phenylbenzopyrone and differing one from another in the pattern of hydroxylation, degree of unsaturation and type and position of sugar links. Although the flavonoids are weak acids (phenols), they are best separated on the basis of their hydrophobic differentiation, that is, by reversed-phase (RP) HPLC. CE in the micellar mode pro-

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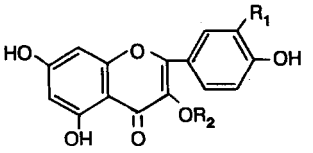
vides an alternative to RP-HPLC, as in a micellar system ionic detergents are added directly to the mobile phase buffer to generate aggregates consisting of a hydrophobic interior and a charged surface.

Extending the previous study [8] on developing methods for the analysis of flavonoid drugs, micellar electrokinetic capillary chromatography (MECC) was used for the separation of a number of flavonol-3-O-glycosides which are commonly present in medicinal plants. This approach represents a new and powerful tool, as it allows a rapid, reproducible, high resolution and sensitive determination of flavonol-3-O-glycosides, as confirmed by the results presented in this paper.

EXPERIMENTAL

Materials

The reference compounds (Fig. 1, I-VII and IX) were obtained from Extrasynthese (Genay, France). Compound VIII was already available in this laboratory [9]. A standardized *Ginkgo biloba* extract was purchased from Laboratories IPSEN.



	R_1	R_2
Rutin (I)	OH	Rutinose
Isoquercitrin (II)	OH	Glucose
Hyperosid (III)	OH	Galactose
Quercitrin (IV)	OH	Rhamnose
Avicularin (V)	OH	Arabinose
Kaempferol-3-O-rutinoside (VI)	H	Rutinose
Isorhamnetin-3-O-rutinoside (VII)	OMe	Rutinose
Astragalin (VIII)	H	Glucose
Isorhamnetin-3-O-glucoside (IX)	OMe	Glucose

Fig. 1. Structures of the flavonol-3-O-glycosides.

All the other chemicals were of HPLC grade.

Apparatus

MECC. All separations were performed using an Applied Biosystems 270A CE apparatus (San Jose, CA, USA) equipped with a 72-cm fused silica capillary column (50 μm I.D.). The analysis buffer was 20 m*M* sodium borate (pH 8.3) and 50 m*M* sodium dodecyl sulphate (SDS). The other conditions were: voltage, +20 kV (+277 V/cm); temperature, 27°C; injection, 1 s aspiration (4 nl); and detection, 260 nm. All buffers were filtered using a Sigma (St. Louis, MO, USA) 0.2 μm filter. To maintain the capillary conditions, fresh buffer was introduced into the capillary between each run. This was achieved by a 2 min (approximately two volumes) aspiration of the running buffer.

The data were collected and analysed on a Shimadzu CR6A data processor (Kyoto, Japan).

HPLC. The liquid chromatograph consisted of a Model U6K universal in-

jector, two Model 510 pumps, a Model 680 automated gradient controller and a Model 480 Lambda-Max UV variable-wavelength detector (all from Waters Assoc., Milford, MA, USA). The data were collected and analysed on a Shimadzu CR3A data processor (Shimadzu, Kyoto, Japan). The column was a C_8 Aquapore RP-300 (7 μm spherical cartridge, 220 × 4.6 mm I.D.) with an Aquapore RP-300 guard column (7 μm spherical cartridge, 30 × 4.6 mm) from Applied Biosystems. Eluent A was water–2-propanol (95:5) and eluent B 2-propanol–tetrahydofuran(THF)–water (40:10:50). The gradient profile was linear from 20 to 60% B in 40 min at a flow-rate of 1 ml/min; detection was at 260 nm.

Calibration graphs

Compounds I–IX were dissolved in methanol (0.15 mg/ml); these stock solutions were diluted with 30% aqueous methanol to obtain reference solutions containing 2–250 μg/ml.

Sample preparation

The standardized *Ginkgo biloba* extract (5 mg) was dissolved in 1 ml of 30% aqueous methanol and filtered through Spartan 13 filters (0.45 μm, Schleicher and Schuel, Dassel, Germany).

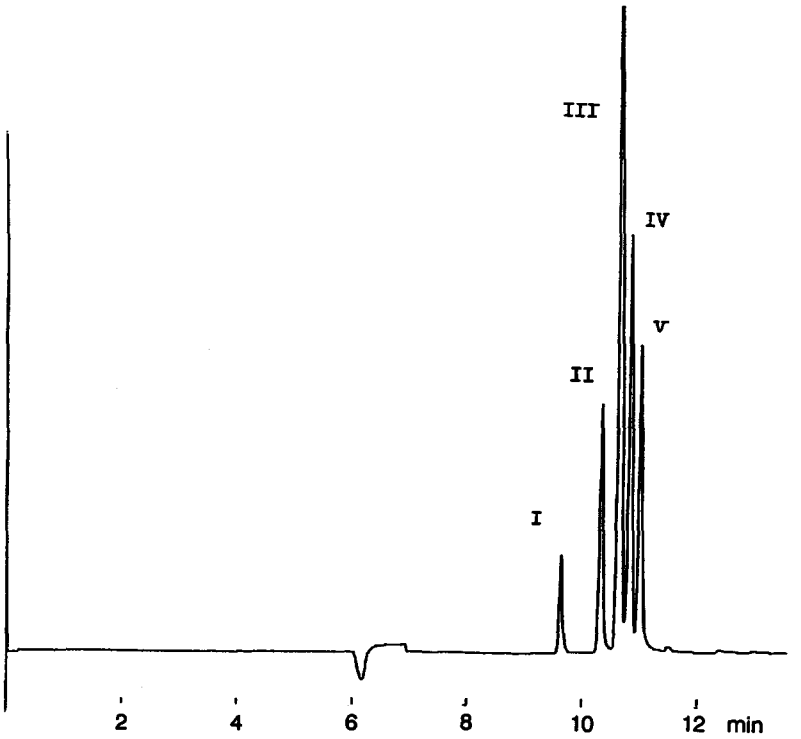


Fig. 2. MECC separation of quercetin-3-O-glycosides (20–100 pg; compounds I–V). See Fig. 1 for peak identification. Conditions: capillary, 72-cm fused silica (50 μm I.D.); voltage, 277 V/cm; buffer, 50 m*M* SDS–20 m*M* borate (pH 8.3); injection, 1 s aspiration; detection, 260 nm; attenuation 16 (mV/full scale).

RESULTS AND DISCUSSION

Of the flavonol-3-O-glycosides the most common are the quercetin, kaempferol and isorhamnetin derivatives (Fig. 1). Their determination has been traditionally carried out by RP-HPLC, whereas electrophoresis finds a limited application in the recognition of charged species, such as sulphates and glucuronides [10]. By using MECC, it has been possible for the first time to determine the flavonol-3-glycosides. According to the MECC technique, SDS was added to the running buffer (50 m*M* SDS–20 m*M* sodium borate, pH 8.3) to generate the micelles. At this pH, the electroendosmotic flow is sufficiently strong to overcome the tendency for negatively charged glycosides to migrate away from the detector, and separation takes place as a result of differences in partitioning into and out of the hydrophobic core of the micelles.

A typical electropherogram of a standard mixture of quercetin-3-O-glycosides (I–IV) is shown in Fig. 2. The five compounds gave well resolved peaks within a 14

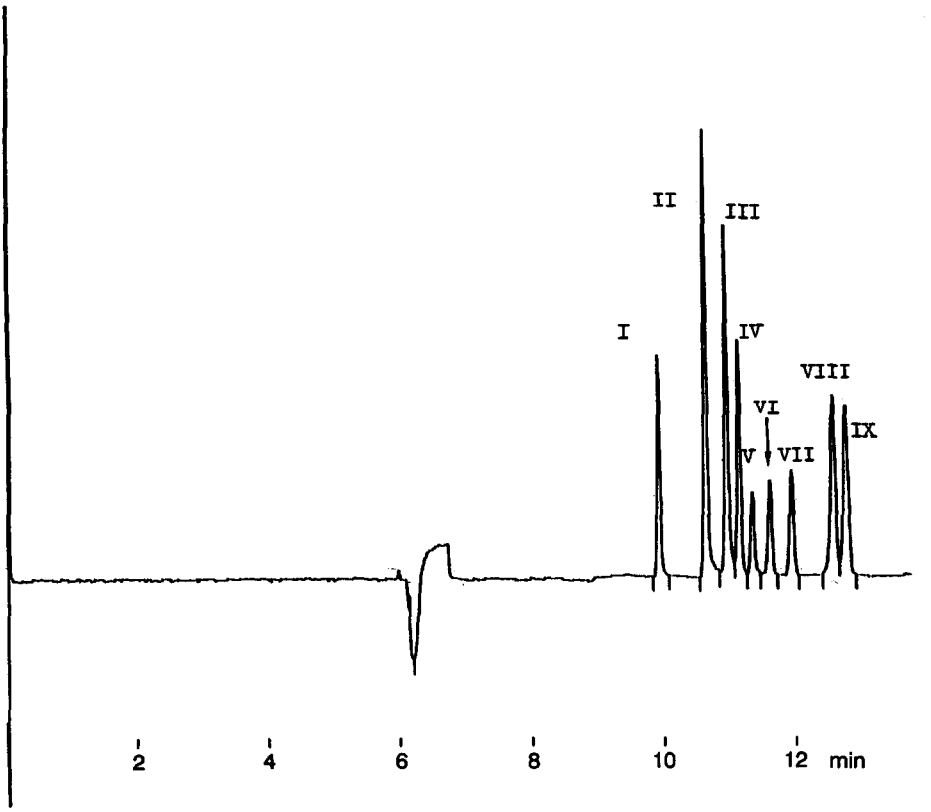


Fig. 3. MECC separation of quercetin-3-O-glycosides (compound I–V), kaempferol-3-O-glycosides (compounds VI, VIII) and isorhamnetin-3-O-glycosides (compounds VII, IX). See Fig. 1 for peak identification. Conditions as in Fig. 2.

TABLE I

MIGRATION (CE) AND RETENTION (HPLC) TIMES

Peak	CE (min)	HPLC (min)
I	9.90	11.76
II	10.61	13.92
III	10.93	13.92
IV	11.13	18.61
V	11.32	15.14
VI	11.61	15.73
VII	11.94	14.57
VIII	12.57	17.74
IX	12.76	16.52

min analysis time, and the standard deviations of the migration times were 0.4% (n = 40) and 0.7% (n = 10) for intra- and inter-day assays, respectively.

To the authors' knowledge, there is no isocratic HPLC system which allows the same separation, and a possible gradient approach is less rapid and simple. Analogous results were obtained for kaempferol- and isorhamnetin-3-O-glycosides. Although their simultaneous occurrence in medicinal plants is not frequent, a mixture of

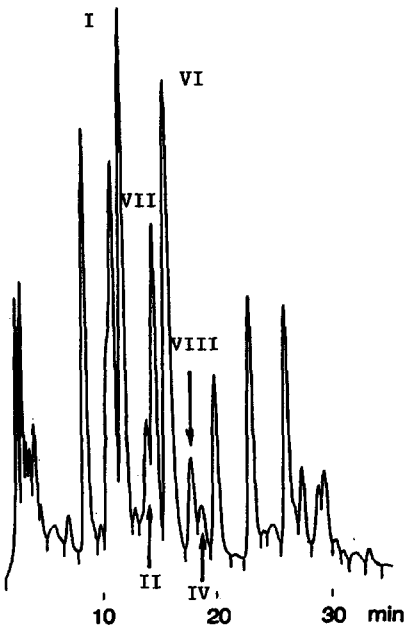


Fig. 4. RP-HPLC separation of standardized extract of *Ginkgo biloba* leaves. Conditions: column, C_8 Aquapore RP-300, 7 μm; eluent A, water–2-propanol (95:5); eluent B, water–THF–2-propanol (50:10:40); gradient profile, from 20 to 60% B in 40 min; flow-rate, 1 ml/min; detection, 260 nm. See Fig. 1 for peak identification.

all the glycosides (I–IX) was also analysed, and a sharp baseline separation was obtained (Fig. 3).

The elution order is slightly different from that found in RP-HPLC, as in addition to the hydrophobic interaction with the micelles, electrophoretic mechanisms are also occurring (Table I).

The detector response was linear over the range 10–1000 pg (15–1500 fmol), the regression coefficient being between 0.995 and 0.999 (n = 20; S.D. = 1.2 and 1.9% for intra- and inter-day, assays, respectively).

By injecting 4 nl of the sample solutions (1.5 μg/ml) in 30% methanol, amounts as low as 6 pg were detected. The percentage of organic solvent was of prime importance, as higher concentrations resulted in a poor resolution.

To confirm its validity, this technique was applied to a particularly complex matrix, *i.e.* to *Ginkgo biloba* extract, the analysis of which by RP-HPLC (Fig. 4) in the gradient mode has been previously reported [11]. As shown in Fig. 5, MECC yielded a high baseline resolution within 14 min. Of the peaks, I, II, IV, VI, VII and VIII were identified by comparison with standard.

From these results, it can be reasonably deduced that MECC is a promising tool for the determination of flavonoids. It has a greater resolving power and is faster than RP-HPLC. The level of sensitivity compared to HPLC (about 50 ng) is also

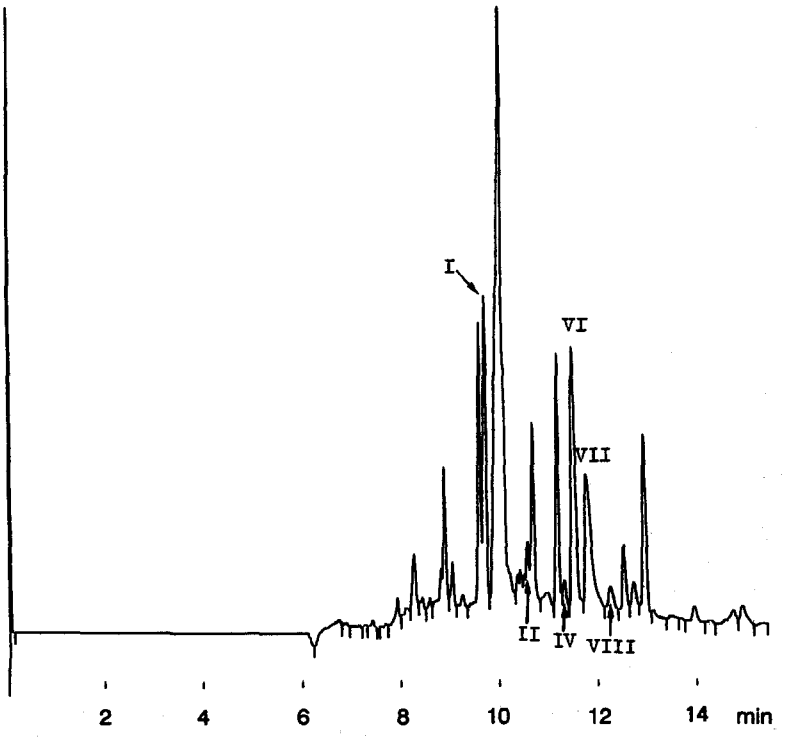


Fig. 5. MECC separation of a standardized extract of *Ginkgo biloba* leaves. For peak identification see Fig. 1. Conditions as in Fig. 2.

higher; in addition, MECC has the advantages of very low solvent consumption and the data analysis capabilities of HPLC. Owing to its complementary value with RP-HPLC, the described procedure will be extended to other flavonoids (flavones, isoflavones, flavanoids, biflavones, anthocyanins and proanthocyanins) with the aim of developing an MECC based 'finger printing' technique for medicinal plants.

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