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Short Communication

HPLC-Analysis of Fumarates in Biological Matrices[#]

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Summary. Simple isocratic HPLC systems without tedious purification steps are elaborated for analyses of fumaric acid, its dimethyl and monomethyl ester, useful for analyses in biological matrices.

Keywords. Isocratic HPLC; Fumaric acid; Dimethyl fumarate; Monomethyl fumarate; Biological matrices.

Introduction

Fumaric acid esters have been used in the treatment of psoriasis for decades. They proved to be effective in patients with chronic plaque type psoriasis according to the results obtained in multicentre trials [1, 2]. Many papers have been published showing the interference of fumarates with cells and their biochemical compartments important for the expression of psoriatic symptoms. Thus, *Höxtermann et al.* [3] have examined the immunological changes in patients suffering from psoriasis who have been treated with esters of fumaric acid. *Ockenfels et al.* [4] have found that dimethyl fumarate diminishes IL-6 and TGF-α secretion in cultures of keratinocytes, co-cultured with HUT 78 T cells (Human Lymphoma T cell line), whereas this effect has not been observed in monocultures of keratinocytes. The authors report upon an immunomodulation from TH1 cytokine interferon γ to the TH2 cytokine IL-10 and conclude that this modulation could be responsible for the beneficial effect of dimethyl fumarate in the treatment of psoriasis.

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Results and Discussion

It is reasonably hypothesized that fumarates are hydrolyzed under physiological conditions and that fumaric acid so obtained is degraded in the citrate cycle. HPLC methods for fumaric acid and fumarates have been published *inter alia* by *Tusseau et al*. [5], *Reddingius* [6], *Negrusz et al*. [7], and *Sarzanini et al*. [8]. Because in our hands various methods for HPLC separation of fumaric acid and its esters in one run or in one system, resp., failed, the methods described below (Experimental) had to be developed proceeding from the method of *Reddingius*. We encountered the following problems: matrices were not homogeneous and contained varying amounts of proteins; there were significant risks of absorption and co-elution, and many compounds absorbed UV light of 215 nm. When analyzing intracellular fluids, the samples are rather small and the concentrations of fumarates are low. Impeding factors also resulted from the analytes: the fumarates have low absorption coefficients at 215 nm and their retention times are near those of matrix components. Under usual reversed phase HPLC conditions, very low capacity factors are commonly achieved, unless ion-suppression LC [5], ion-exchange [8], or ion-pair LC were used. Moreover, there is a lack of functional groups in these analytes suitable for derivatization in order to increase the detection limit.

As one-run simultaneous determination of fumaric acid and its esters was not possible in an isocratic system, we had to employ two different isocratic HPLC systems (Experimental), when analyzing fumaric acid and its esters in biological samples in order to avoid interference of the background. The first one uses ion-suppression in order to increase the retention of the solutes which are kept uncharged. In the second one, ion-pairs between the negatively charged analytes

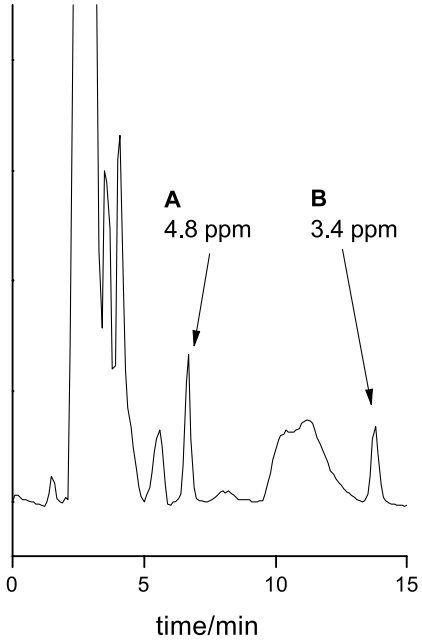

Fig. 1. HPLC analysis of an extract of keratinocytes, treated with dimethyl fumarate (Experimental); **A**: monomethyl fumarate, t_R: 6.8 min; **B**: dimethyl fumarate, t_R: 13.0 min

and the tetrabutylammonium cations are formed. These systems were tested for their usefulness to study the hydrolysis of dimethyl fumarate and monomethyl fumarate in buffer of physiological *pH*-value and in biological matrices. Here, we used serum and human keratinocytes as test systems (Experimental).

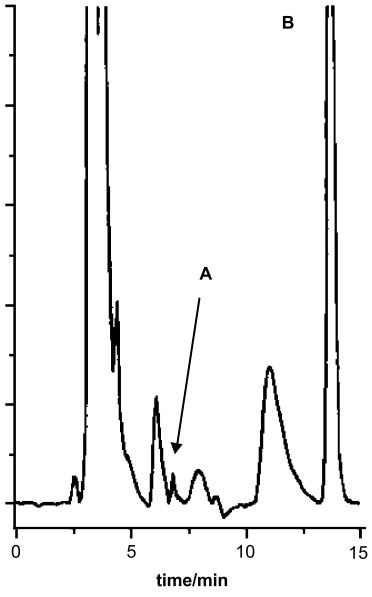

Fig. 2. HPLC analysis of the supernatant of sonicated keratinocytes (Experimental); **A**: monomethyl fumarate, t_R: 6.8 min; **B**: dimethyl fumarate, t_R: 13.0 min

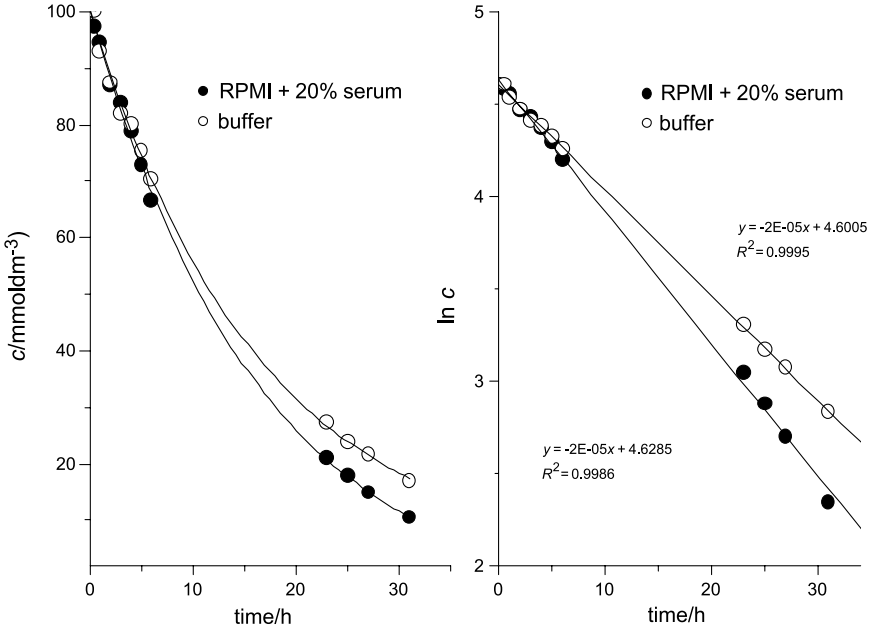

Fig. 3. Hydrolysis of dimethyl fumarate in buffer and in RPMI with 20% fetal calf serum, resp., affording monomethyl fumarate; right: hydrolysis curves in logarithmic scale, fitted by linear regression

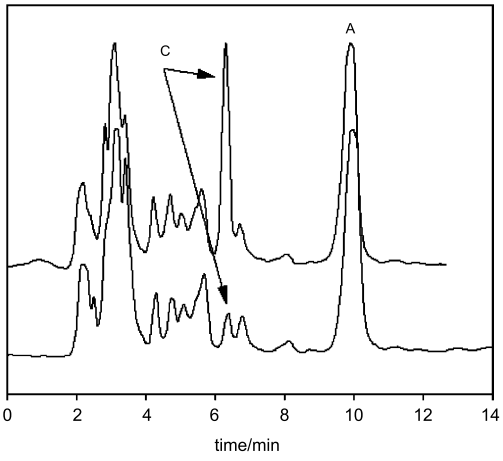

Fig. 4. Ion-pair LC of fumaric acid **C** and monomethyl fumarate **A** in an extract of keratinocytes; upper chromatogram: the same sample spiked with standards of **C** and **A**, resp., for identification; t_R of **C**: 6.3 min; t_R of **A**: 10.0 min

A typical chromatogram of an extract of keratinocytes after treatment with dimethyl fumarate is shown in Fig. 1.

Dimethyl fumarate is hydrolyzed in the cells affording monomethyl fumarate, whereas in the supernatant only a minimal amount of monomethyl fumarate can be found (Fig. 2).

Figure 3 shows the hydrolysis of dimethyl fumarate in RPMI cell culture medium containing 20% fetal calf serum and in buffer.

Figure 4 represents an ion-pair LC of fumaric acid and monomethyl fumarate in a cell extract; the peaks are identified by spiking (upper part of the graph). These methods can now be used by biochemists and immunologists in the context of their investigations.

Conclusion

Our HPLC methods are suitable for the determination of fumarates in biological matrices; our results indicate that dimethyl fumarate is rapidly hydrolyzed yielding monomethyl fumarate, whereas further hydrolysis affording fumaric acid is far slower than the first step.

Experimental

Dimethyl fumarate and monomethyl hydrogen fumarate were obtained from Fumapharm, Luzern, Switzerland. Their purities (>98%) were determined by the HPLC systems described below. Acetonitrile, methanol, sodium dihydrogen phosphate, phosphoric acid (85%), and tetrabutylammonium hydrogen sulfate were purchased from Fluka (Buchs, Switzerland) and were of HPLC (acetonitrile, methanol) or analytical grade. Liquid RPMI-1640 medium and liquid Minimum Essential Medium Eagle (MEM) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Standard solutions proved not to be stable. Therefore, stock solutions of the standards for calibration curves were prepared directly before use. Re-distilled water was used in all cases. Hydrolysis of dimethyl fumarate was

studied in phosphate buffer *pH* 7.3 [9], in RPMI containing 20% fetal calf serum, and in keratinocytes.

HaCaT keratinocytes were incubated with dimethyl fumarate 10 $\mu g/cm^3$ for 30 min in MEM medium with 10% fetal calf serum. Dimethyl fumarate was dissolved in dimethyl sulfoxide to a stock concentration of 10 mg/cm^3 and further diluted to the final concentration using MEM fetal calf serum.

For dimethyl and monomethyl fumarate ion-suppression reversed-phase LC was used, whereas fumaric acid and monomethyl fumarate were determined by ion-pair chromatography (IPC). The solutions were kept at 37°C (thermostated water bath). Samples were withdrawn in given time steps and directly injected into the HPLC system.

HPLC: Kontron 420 HPLC pump (Kontron Instruments, Eching, Germany), equipped with a tunable Kontron 430 HPLC UV detector. Samples were transferred to the column (see below) by a Rheodyne 7121 injector (Rheodyne, Cotati, CA, USA) with a 20 mm^3 loop, using a glass 50 mm^3 Hamilton syringe (Hamilton, Reno, NV, USA). The column was equipped with a LiChrospher 100 RP-18 pre-column in a steel cartridge (ManuCart, Darmstadt, Germany). Data were recorded at 215 nm using a MacLab/4 AD LC interface, coupled with a MacIntosh Quadra 700 computer with a Chart/4 v.3.1.3 and Peaks v.1.1 ß 1 LC software.

HPLC conditions for dimethyl and monomethyl fumarate: Column: LiChrosorb C 18, 5 μm, 250 × 4 mm ID. Mobile phase: 0.01 *M* H_3PO_4/*Me*OH (55/45 *v*/*v*). Flow-rate: 1.0 cm^3/min. Retention times: monomethyl fumarate 6.8 min, dimethyl fumarate 13.6 min.

HPLC conditions for fumaric acid and monomethyl fumarate: same column. Mobile phase: 50 m*M* phosphate buffer [9] *pH* 5.5 with an aqueous solution of 25 m*M* tetrabutylammonium hydrogen sulfate/acetonitrile (95/5 *v*/*v*). Flow-rate: 1.0 cm^3/min. Retention times: fumaric acid 6.3 min, monomethyl fumarate 10.0 min.

Cell Sample Preparation

The frozen keratinocytes, as obtained from the Department of Dermatology of the University of Kiel, Germany, were left at ambient temperature to thaw. Then, 100 mm^3 of acetonitrile and 100 mm^3 of water were added, followed by sonication in an ice bath for 2 min with an Ultraturrax ultrasonic stick pulse modus. The mixture was transferred into a 0.5 cm^3 plastic vial and centrifuged (20 min at 7 200 G). The supernatants were removed and directly injected without further purification.

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