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Short communication

Postcolumn derivatisation for selective laser-induced fluorescence detection in micellar electrokinetic chromatography of fatty acid hydroperoxides

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Abstract

A new method of postcolumn derivatisation in capillary electrophoresis is demonstrated by the reaction of fatty acid hydroperoxides with *p*-hydroxyphenylacetic acid (HPAA) to give the fluorescent 'dimer' of HPAA for laser-induced fluorescence detection. This reaction is catalysed by microperoxidase-11 covalently immobilised in a reaction capillary that is either spliced or butt joined onto the end of the separation capillary. The micellar electrokinetic chromatography of the isomeric hydroperoxides formed by photo-oxidation of oleic, linoleic and linolenic acids shows that the derivatisation reaction does not lead to significant broadening of the peaks.

Keywords: Derivatisation, electrophoresis; Fatty acid hydroperoxides; Hydroxyphenylacetic acid

1. Introduction

Most groups analysing for lipid hydroperoxides by HPLC have chosen postcolumn reactions to increase the sensitivity and selectivity of detection. A common method is the measurement of the chemiluminescence resulting from the reaction of hydroperoxides with the combination of microperoxidase-11 (MP-11) and either luminol or isoluminol [1,2], which leads to detection limits in the low μM range. Heinmöller et al. achieved selective and sensitive fluorescence detection of lipid hydroperoxides by using MP-11 and *p*-hydroxyphenylacetic acid (HPAA) in the postcolumn reaction [3]; coupling with RP-HPLC allowed the detection of lipid hydroperoxides in aged edible oils and in tobacco plants treated with ozone gas [4]. After hydrolysis or transesterification of these lipids, the groups of hydroperoxides from oleic, linoleic and linolenic acids (as acids or methyl esters) could be separated by RP-HPLC, but the isomeric hydroperoxides of the individual acids could not.

We have recently shown that micellar electrokinetic chromatography (MEKC) with micelles of polyoxyethylene lauryl ether (Brij 35) or mixed micelles of Brij 35 and sodium dodecyl sulphate (SDS) [5,6], coupled with UV-diode-array detection (UV-DAD), is a powerful method for the analysis of the isomeric hydroperoxides derived from oleic (18:1-HP), linoleic (18:2-HP) and linolenic (18:3-

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HP) acids. Since UV-DAD is susceptible to interferences in the analysis of these hydroperoxides in biological samples with more complex matrices, we have now extended to CE the highly selective HPLC postcolumn reaction with MP-11/HPAA and fluorescence detection.

For postcolumn derivatisation in CE, the reagent solution is commonly introduced to the electrolyte through a tee, in which the separation capillary is inserted coaxially into a larger reaction capillary [7–11]. The space around the smaller capillary allows the reagent to flow in. Disadvantages of this method are the difficulty of inserting and fixing the capillaries [12–14] and some loss of resolution. The systems published so far have the further disadvantage of not fitting into commercial CE instruments that cool the capillaries with a circulating liquid.

In the new method of postcolumn derivatisation described here, we have achieved the reaction of HPAA to the 'dimer' without discernible peak broadening by coupling the capillary for the MEKC separation of the hydroperoxides with a short reaction capillary where MP-11 is covalently immobilised on the wall.

2. Experimental

2.1. Apparatus

A Beckman P/ACE System 5510 with either a UV-DAD system or a laser-induced fluorescence (LIF) detector (He–Cd, λ_{ex}: 325 nm, λ_{em}: 415 nm) was used for the analyses. The separation took place at 25°C in a fused-silica capillary [L=40/47 cm (effective/total length)] or in a fused-silica capillary (L=40 cm) coupled with a reaction capillary (L=10/17 cm) with MP-11 immobilised on the capillary wall. The fused-silica capillaries were from CS-Chromatographie-Service (Langerwehe, Germany) and had an I.D. of 50 μm. The field strength was either 596 V/cm (L_{total}=47 cm) or 491 V/cm (L_{total}=57 cm). Before each run the capillary was rinsed for 2 min with tridest (deionised and doubly distilled) water, 1.5 min with 0.1 *M* HCl, 2 min with tridest water and 3 min with electrolyte. The sample was introduced hydrodynamically (5 s), and the detector was at the anodic end of the capillary.

Two methods were used to join the capillaries. In the first, a variant of a method described as butt joining [15], the capillary ends were smoothed to planarity with fine sandpaper to give a flush joint and simply inserted into a PTFE cylinder (L=7 mm, O.D.=2.5 mm) with a 350 μm axial bore; the softness of the PTFE made it possible to insert capillaries with O.D. of 360 μm. In the second method, they were connected by the use of an optical splicer Model OSG 15 from Felten and Guilleaume (Cologne, Germany), the technique being the same as that employed in splicing optical fibres [16]. A slight pressure of N_2 in the capillaries prevented any change in capillary diameter. Since the polyimide coating of the capillaries is lost in the process, the splice represents a weak point. A PTFE cylinder was slipped over the splice to lend the capillaries more resistance to mechanical stress (in being wound up in the cartridge, for example). In either case, the thin PVC mantle of the cylinder allowed the joint to be stabilised with cyanoacrylate cement.

2.2. Reagents

MP-11 and the fatty acids were from Sigma (Deisenhofen, Germany); all other chemicals were from Merck (Darmstadt, Germany) and of analytical-reagent quality. Tridest water was used for the electrolyte solutions, which were passed through a PTFE filter (0.45 μm) and degassed ultrasonically. Brij 35 and SDS were used as surfactants and ethanol and acetone as organic modifiers.

The reagent for the immobilisation was made by dissolution of 4 mg MP-11 and 35–40 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) in 3 ml of 10 m*M* N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulphonic acid) (HEPES) adjusted to pH 7.8 with 1 *M* NaOH.

2.3. Oxidation of the fatty acids

The hydroperoxides from oleic, linoleic and linolenic acids were prepared as described earlier [5,6,17,18] by treatment of the acids with singlet oxygen and after purification were characterised by NMR. In addition, linoleic and linolenic acids were oxidised enzymatically [19,20] to 13-(*S*)-hydroperoxyoctadeca-9*Z*,11*E*-dienoic (13*S*-18:2-HP, **5**)

and 13(*S*)-hydroperoxyoctadeca-9*Z*,11*E*,15*Z*-trienoic (13*S*-18:3-HP, **7**) acids, respectively.

2.4. Immobilisation

The immobilisation of MP-11 proceeded through the following three steps: etching of the capillary surface by acid, silanisation of the activated surface to introduce primary amino groups and finally covalent bonding of the biological catalyst [21,22]. This method assured that the MP-11 would be attached to the surface only through its carboxyl groups. Specifically, the capillary was rinsed as follows:

2 h with 5% (v/v) HNO_3 at 45°C
1 h with tridest water
8 h at 35°C with a 10% solution of 3-(triethoxysilyl)propylamine in water adjusted to pH 3.5 with 6 *M* HCl
1 h with tridest water
(after being dried overnight at 115°C) 8 h at room temperature with the freshly prepared mixture of MP-11 and EDAC in HEPES buffer
1 h with tridest water

3. Results and discussion

The availability of enzymatically synthesised 13*S*-18:2-HP (**5**) and 13*S*-18:3-HP (**7**) allowed the assignment of the signals of these compounds. The different rates of formation of the two hydroperoxides from the photo-oxidation of oleic acid [23] made clear the assignment of the signals of 10-hydroperoxyoctadec-8*E*-enoic (**1**) and 9-hydroperoxyoctadec-10*E*-enoic (**2**) acids. Further partial assignment was attained through the fact that the conjugated hydroperoxides of linoleic and linolenic acids absorb more strongly at 234 nm than the nonconjugated isomers.

Capillaries with MP-11 immobilised as described are stable to 0.1 *M* HCl and if stored full of HEPES buffer can be used for several weeks.

To determine whether the introduction of a junction of two capillaries would lead to broadening of the peaks, composites of two fused-silica capillaries (L_1=30 cm, L_2=10/17 cm) were prepared by each method, without any immobilised MP-11, and compared with the continuous fused-silica capillary (L= 40/47 cm). There was no discernible broadening of the peaks with either composite capillary.

In earlier studies of MEKC of fatty acid hydroperoxides [6,13] we used an electrolyte containing SDS and Brij 35 with ethanol as modifier. In all the work described here a small amount of acetone was added as well, to increase the resolution. The most successful electrolyte found thus far is a solution containing 140 parts (by volume) of 60 m*M* sodium phosphate buffer (pH 6.3), 60 parts of ethanol and 1 part of acetone, to each litre of which are added 720 mg of Brij 35 and 30 mmol of SDS. Fig. 1, an electropherogram of 18:1-HP (two isomers), 18:2-HP (four isomers) and 18:3-HP (six isomers) with the continuous capillary and diode-array detection, shows the good resolution obtained with this electrolyte. The presence of twice as much acetone in the electrolyte led to a further improvement in the resolution, allowing five of the six hydroperoxides from linolenic acid to be distinguished clearly (continuous capillary, UV-DAD de-

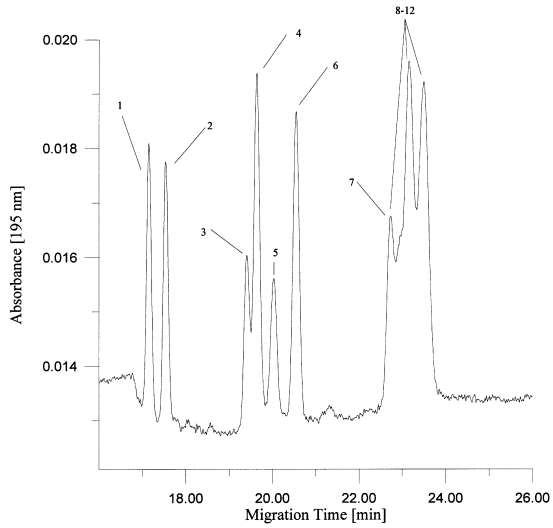

Fig. 1. Electropherogram of a mixture of 18:1-HP (**1**, **2**; 1.1 m*M*), 18:2-HP (**3**–**6**, 1.9 m*M*) and 18:3-HP (**7**–**12**, 1.4 m*M*). Capillary: uncoated fused-silica (L=40/47, 50 μm). Electrolyte: a solution containing 140 parts (by volume) of 60 m*M* sodium phosphate buffer (pH 6.3), 60 parts of ethanol and 1 part of acetone, to each litre of which were added 720 mg of Brij 35 and 30 m*M* of SDS. Field strength: 596 V/cm. Detection: UV-DAD at 195 nm.

tection), but it unfortunately also led to a loss of fluorescence intensity (not pictured).

The electropherograms in Fig. 2, with LIF detection, were made with a composite capillary prepared by the second method (splicing) and containing immobilised MP-11. The HPAA necessary for the postcolumn reaction was added to the electrolyte at 0.4 m*M*; increasing the concentration did not lead to any further increase in the fluorescence. Fig. 2 makes it clear that the postcolumn derivatisation does not lead to a loss of resolution. Indeed, the resolution is somewhat better in Fig. 2, but this is probably an effect of the decreased field strength (the capillary used to obtain the electropherogram in Fig. 2 is 10 cm longer).

Resolution similar to that of Fig. 2 was obtained by postcolumn reaction with the composite capillary made by insertion of capillary ends into a PTFE cylinder (the first method), but there was a slight downward drift of the baseline (not shown).

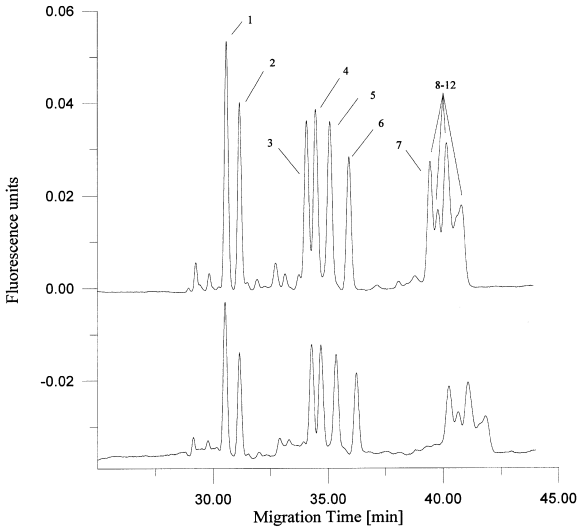

Fig. 2. Electropherograms of a mixture of 18:1-HP (**1**, **2**; 1.1 m*M*), 18:2-HP (**3**–**6**, 1.9 m*M*) and 18:3-HP (**7**–**12**, 1.4 m*M*), with and without acetone in the outlet vial. Capillary: composite of uncoated fused-silica (L=40, 50 μm) and a reaction capillary (L=10/17, 50 μm) containing immobilised MP-11. Electrolyte (lower electropherogram): a solution containing 140 parts (by volume) of 60 m*M* sodium phosphate buffer (pH 6.3), 60 parts of ethanol and 1 part of acetone, to each litre of which were added 720 mg of Brij 35 and 30 m*M* of SDS. Electrolyte (upper electropherogram): as above but without acetone in outlet vial. Field strength: 491 V/cm. Detection: LIF detector (He–Cd, λ_{ex}: 325 nm, λ_{em}: 415 nm).

The upper electropherogram in Fig. 2 shows that significantly greater intensity of fluorescence is achieved with a system where acetone is present only in the electrolyte of the inlet end. The origin of this effect is not yet known. However, since the electroosmotic flow (EOF) is directed toward the inlet (cathode), the electrolyte in the outlet vial, without any acetone, is drawn into the reaction capillary. This may suppress some deleterious effect of acetone on either the postcolumn reaction or the LIF detection itself.

The detection limit of the hydroperoxides was determined to be 10–15 μ*M* (3σ), which is lower by a factor of ten than that of the nonconjugated isomers at 195 nm [13] with UV-DAD detection and in the same range as that of the conjugated hydroperoxide isomers at 234 nm. In addition to the low detection limit for analytes with weak chromophores, the method achieves a very high selectivity, since the highly fluorescent compound detected is produced only from the hydroperoxides.

4. Conclusions

MEKC with the selective postcolumn reaction of MP-11 immobilised in a composite capillary was used here to analyse fatty acid hydroperoxides with high resolution and good sensitivity. Such a CE capillary can be made efficiently enough by at least two methods that the joint does not lead to significant loss of resolution. Since enzyme reactions are used in a wide variety of detection systems nowadays, the method described here with MP-11 must be viewed as only the first example of a technique that can be applied to other classes of compounds.

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References

[1] Y. Yamamoto, M.H. Brodsky, J.C. Baker, B.N. Ames, Anal. Biochem. 160 (1987) 7–13.
[2] B. Frei, Y. Yamamoto, D. Niclas, B.N. Ames, Anal. Biochem. 175 (1988) 120–130.
[3] P. Heinmöller, H.-H. Kurth, R. Rabong, W.V. Turner, A. Kettrup, S. Gäb, Anal. Chem. 70 (1998) 1437–1439.
[4] P. Heinmöller, Doctoral Dissertation, Technische Universität München, Munich, 1997.
[5] O. Schmitz, S. Gäb, J. Chromatogr. A 767 (1997) 249–253.
[6] O. Schmitz, S. Gäb, J. Chromatogr. A 781 (1997) 215–221.
[7] D.J. Rose Jr., J.W. Jorgenson, J. Chromatogr. 447 (1988) 117–131.
[8] B. Nickerson, J.W. Jorgenson, J. Chromatogr. 480 (1989) 157–168.
[9] A. Emmer, J. Roeraade, J. Chromatogr. A 662 (1994) 375–381.
[10] T.T. Lee, R. Dadoo, R.N. Zare, Anal. Chem. 66 (1994) 2694–2700.
[11] L. Zhang, E.S. Yeung, J. Chromatogr. A 734 (1996) 331–337.
[12] A. Rosenstock, Master's Dissertation, Bergische Universität-Gesamthochschule, Wuppertal, 1995.
[13] O. Schmitz, Doctoral Dissertation, Bergische Universität-Gesamthochschule, Wuppertal, 1997.
[14] H.A. Bardelmeijer, J.C.M. Waterval, H. Lingeman, R. van 't Hof, A. Bult, W.J.M. Underberg, Electrophoresis 18 (1997) 2214–2227.
[15] M.E.P. Hows, D. Perrett, Chromatographia 43 (1996) 200–204.
[16] C.M. Miller, Techn. Digest Opt. Fiber Transmission II (1977) WA3-1-3-6.
[17] E.N. Frankel, Prog. Lipid Res. 19 (1980) 1–22.
[18] E.N. Frankel, Prog. Lipid Res. 23 (1985) 197–221.
[19] M.O. Funk, J.C. Andre, T. Otsuki, Biochemistry 26 (1987) 6880–6884.
[20] H.W. Gardner, Biochim. Biophys. Acta 1001 (1989) 274–281.
[21] H. Weetall, Biosensors Bioelectronics 8 (1993) x–xi.
[22] T. Lötzbeyer, W. Schuhmann, H.-L. Schmidt, J. Electroanal. Chem. 377 (1994) 291–294.
[23] H.-D. Belitz, W. Grosch, Lehrbuch der Lebensmittelchemie, Springer, Berlin, 1992.