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Review

Determination of free radical reaction products and metabolites of salicylic acid using capillary electrophoresis and micellar electrokinetic chromatography

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Abstract

Hydroxylated radical products of salicylic acid are often used as a relative measurement in free radical research. Several analytical methods exist to determine the amount of 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid. In this study we use capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC) in order to determine these free radical products. The CZE experiment was optimized with a CZE simulation program HPCESIM in order to achieve an optimal pH. Calibration curves were recorded in the range 10^{-6}–10^{-4} *M* and the detection limit was determined. For both CZE and MECC it was $2 \cdot 10^{-7}$ *M*. Both methods resulted in a reproducible analysis of salicylate and its hydroxylated free radical products in 6 min.

Keywords: Reviews; Salicylic acid; Free racicals

Contents

1. Introduction

In the research field of free radicals in biological samples it still is a major problem to determine the amount of free radical damage. One of the most aggressive radicals is the hydroxyl radical. Consequently, a good analytical method is necessary to determine the extend of free radical damage in vivo, caused by hydroxyl radicals.

Several methods exist to quantify the damaging effect of these radicals. Most of the methods use endogenous markers to determine this damage, e.g.,

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the determination of pentane and butane in the human breath [1–3], the state of lipidperoxydation [4–7] and DNA adducts (e.g., 8-hydroxydeoxyguanosine) [8–10]. A review on ''the measuring of oxidative stress in vivo'' is given by Hageman et al. [11]. The most commonly used test determines the amount of malondialdehyde formed during oxidative stress [12,13]. However Draper et al. [14] and Cherif et al. [15] showed that the amount of oxidative damage can be misinterpreted if malondialdehyde is used as a marker. The major disadvantage of using endogenous markers in vivo is the possibility that they are not only formed by free radicals but also by other pathways e.g., enzymatically.

Another approach is the measurement of free radical damage via radical trapping by exogenous markers. A method that is often used in free radical research is the measurement of hydroxylated free radical products of salicylic acid. Salicylate has good properties to act as an in vivo marker for oxidative stress. It has a high reaction rate constant with hydroxyl free radicals, $5 \cdot 10^{9}$–10^{10} l mol^{-1} s^{-1} [16], it does not occur in humans, and at least one of the free radical products [2,3-dihydroxybenzoate (2,3-DHB)] is not a natural metabolite of salicylic acid. 2,3-DHB, once formed cannot be further metabolised [17]. When salicylate is exposed to hydroxyl radicals in vitro, three reaction products are formed: catechol (11%), 2,3-DHB (49%) and 2,5 dihydroxybenzoate (2,5-DHB) (40%) (see Fig. 1) [18].

Several researchers used the absolute amount of 2,3-DHB and 2,5-DHB to measure the amount of free radical damage [18,19]. McCabe et al. [20] however pointed out that the proper way to measure the free radical damage is to determine the 2,3-DHB/salicylic acid and 2,5-DHB/salicylic acid ratios. The reason for this is the interindividual difference in biological availability, distribution and metabolism of salicylate. The accuracy and location of salicylate administration can also play a role.

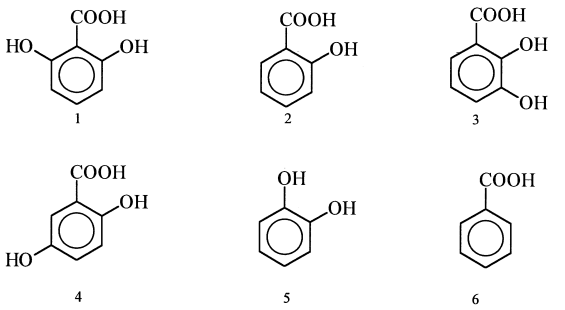

Fig. 1. Salicylic acid (2) and its hydroxylated derivatives 2,5-dihydroxybenzoic acid (4), 2,3-dihydroxybenzoic acid (3), catechol (5), 2,6-dihydroxybenzoic acid (1), benzoic acid (6).

In most of the research, aspirin (*o*-acetylsalicylate) instead of salicylate is administered. Aspirin is hydrolysed rapidly to salicylate in vivo [21,22]. Sixty percent of salicylic acid remains unchanged in the human body and can undergo free radical attack. According to Coudray et al. [23], the maximum salicylate concentration in plasma is reached 0.5–2 h after oral intake of 1000 mg of aspirin. They determined the concentration of 2,3-DHB in plasma as well as in synovial fluid. The concentrations were 230 n*M* and 240 n*M*, respectively.

Different analytical techniques have been used to determine salicylate and its free radical products (2,3-DHB and 2,5-DHB) in vivo. Gas chromatography–mass spectrometry (GC–MS) [24] and high-performance liquid chromatography (HPLC) with electrochemical detection (ED) [25–27] are the most commonly used methods. The sensitivity in HPLC increases a factor 1000 if ED is used instead of a UV spectrophotometer. For the determination of salicylic acid, a HPLC method with UV detection is described in literature that does not require an extraction step before analysis [18]. Some authors describe the use of different methods for the determination of 2,3-DHB, 2,5-DHB and salicylic acid. The disadvantage is that it is necessary to use an internal standard. McCabe et al. [20] describe a method that can be used to determine 2,3-DHB, 2,5-DHB and salicylic acid in one HPLC run. Since the ratios of 2,3-DHB/salicylic acid and 2,5-DHB/salicylic acid are determined, no internal standard is needed. Separation of the analytes was achieved on a reversed-phase column. Analytes were detected on a dual electrode analytical cell with the first electrode set to oxidise the DHBs at +250 mV (vs. Pd) and the second electrode set to oxidise salicylate at +750 mV (vs. Pd) to oxidise contaminants in the mobile phase. Kaur and Halliwell [28] analysed DHBs and salicylate in human plasma in a one electrode system. Their method however involves a complicated sample clean-up procedure and resulted in a complex chro-

matogram with multiple detector sensitivity changes within the chromatographic run.

Recently also a method was described using micellar electrokinetic capillary chromatography (MECC) to determine salicylic acid, 2,5-DHB and 2,3-DHB [29]. In this paper we describe a capillary electrophoretic as well as a micellar electrokinetic capillary chromatographic method to determine the free radical products and the metabolic products of salicylic acid. Both methods will be compared.

2. Experimental

2.1. Materials

Sodium dodecyl sulphate (SDS) (>99%), salicylic acid, 2,5-dihydroxybenzoic acid (2,5-DHB), catechol (>99%) and tris(hydroxymethyl)aminomethane (Tris) (reagent grade) were obtained from Sigma (St. Louis, MO, USA). Sodium tetraborate·$10H_2O$ (>99.5%), boric acid (p.a.), benzoic acid (p.a.) and formic acid (98–100%) were obtained from Merck (Darmstadt, Germany). 2,3-Dihydroxybenzoic acid (2,3-DHB) (>97%) was obtained from Fluka (Buchs, Switzerland).

Because of the instability of dihydroxybenzoic acids [30,31] standard solutions were freshly made everyday.

2.2. Apparatus

All experiments were performed on a P/ACE 5500 capillary electrophoresis (CE) system (Beckman, Fullerton, CA, USA). The capillary was an untreated fused-silica capillary (J&W Scientific, Folsom, CA, USA), 270 mm (length to detector 200 mm)×75 μm I.D. Data acquisition was performed with P/ACE 3.0 software, peak integration with Caesar 4.1 software.

2.3. Operation conditions

The injection time was 10 s, the injection pressure was 3.3 kPa. The UV detector was operated at 200 nm. The capillary was rinsed for 1 min with 0.1 *M* NaOH and for 1 min with the running buffer prior to analysis.

For the capillary zone electrophoresis (CZE) experiments the applied voltage was −20 kV. The running buffer for the CZE experiments consisted of 10 m*M* Tris adjusted to pH 2.78 with formic acid.

For the MECC experiments the applied voltage was 10 kV. The buffer for the MECC experiments consisted of 50 m*M* SDS, 10 m*M* sodium tetraborate and was adjusted to pH 8.4 with boric acid.

3. Results and discussion

Considering the mobilities of the sample components (see Table 1) it is obvious that separation between 2,5-DHB and 2,3-DHB cannot be expected at high pH, where the sample components are fully dissociated. The difference between their pK_a values is only 0.04 (see Table 1). This small difference requires a good estimation of the pH of the buffer that is used, in order to get an optimised separation. In this study a CZE simulation program HPCESIM [32,33] was used to achieve this. Fig. 2 shows an electropherogram of a simulation of the separation of salicylate and its hydroxlyated derivatives. The pH of the buffer used for the separation of salicylate and its hydroxylated derivatives was optimised to 2.78. The importance of the carrying out the simulation before the real experiment could be seen by lowering the pH. At pH 2.6 there was no resolution left between salicylate and 2,3-DHB. Lowering the pH further a reversed peak order could be shown with the optimised separation at pH 2.2. The resolution was less than the resolution at pH 2.8. Separation was achieved between the internal standard, salicylic acid, 2,5-DHB and 2,3-DHB within 6 min. If we compare the simulated electropherogram (Fig. 2)

Table 1
pK_a values [36], octanol–water partition coefficients (P_{ow}) and mobilities (m) of sample components (ionic strength zero, 25°C)

	pK_{a1}	Log (P_{ow})	m (m^2/V s)
Salicylic acid	3.11	2.24	$-35.4\cdot10^{-9}$
2,3-Dihydroxybenzoic acid	2.94	1.15	$-26.6\cdot10^{-9}$
2,5-Dihydroxybenzoic acid	2.98	1.76	$-26.2\cdot10^{-9}$
Catechol	9.84	1.03	–
2,6-Dihydroxybenzoic acid	1.3	1.76	$-37.2\cdot10^{-9}$
Benzoic acid	4.17	1.87	$-33.7\cdot10^{-9}$

The P_{ow} values were calculated with the computer program LOGKOW [37].

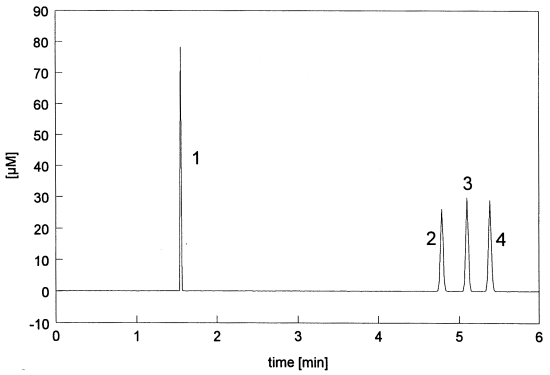

Fig. 2. Simulated electropherogram of mixture of 2,6-DHB (1, I.S.), salicylic acid (2), 2,3-DHB (3) and 2,5-DHB (4). The concentration of each compound was $2\cdot10^{-5}$ *M*. The concentration of the internal standard (I.S.) was $5\cdot10^{-5}$ *M*. Analytical conditions are as described in Section 2.3.

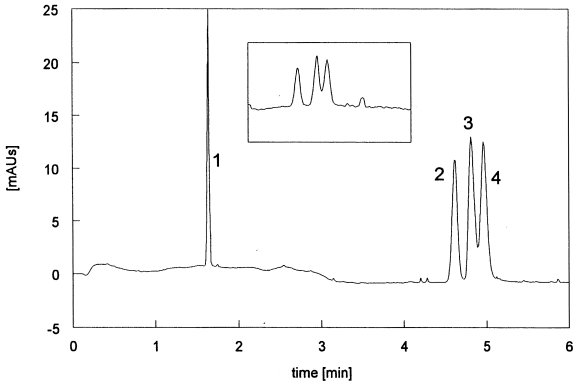

Fig. 3. CZE electropherogram of mixture of 2,6-DHB (1, I.S.), salicylic acid (2), 2,3-DHB (3) and 2,5-DHB (4). The concentration of each compound was $2\cdot10^{-5}$ *M*. The concentration of the internal standard (I.S.) was $5\cdot10^{-5}$ *M*. Analytical conditions are as described in Section 2.3. The inlay shows an electropherogram of a 10^{-6} *M* mixture of the same composition.

with the recorded electropherogram (Fig. 3) we see a good agreement. The resolution however is higher in the simulated electropherogram. The wavelength of the detector was set at 254 nm, 214 nm and 200 nm. A wavelength of 200 nm gave the best signal-to-noise ratio. The detection limit, determined as 4-times the signal-to-noise ratio, was $2\cdot10^{-7}$ *M*.

A linear calibration curve was recorded from 10^{-6} *M* to 10^{-4} *M*. 2,6-DHB was used as an internal standard, concentration $5\cdot10^{-5}$ *M*. The correlation coefficient of all sample components was 0.999. The slope and intercept of the calibration curves for the different sample components are shown in Table 2. The slope (a_1) was determined as dlog(y)/dlog[sample]. For all the samples in the CZE experiments the slope lies between the outer borders of the 95% confidence interval, expecting a slope of 1. The intercept (a_0) was determined from the linear calibration curve.

The equipment used for the CZE experiments can also be used for MECC experiments. Since MECC and CZE are orthogonal techniques, the possibility exists that a better separation could be achieved with MECC. Another advantage of MECC is the possibility to separate neutral compounds like catechol, one of the enzymatically formed metabolites of salicylate.

In these experiments several buffers were used. The best results were achieved with a 10 m*M* sodium tetraborate acidified to pH 8.4 with boric acid and 50 m*M* SDS. This system results in a good separation of all components in 6 min (see Fig. 4). The retention order is completely different from the migration order in CZE, to be expected with orthogonal separation mechanisms. It has been pointed out in the literature that the octanol–water partition coefficient has some predictive possibility for MECC

Table 2
Slope a_1 [dlog(y)/dlog(x)] and linear intercept a_0 and their 95% confidance interval (C.I.) of the calibration line of salicylic acid and its free radical products

Name	CZE		MECC	
	$a_1 \pm$95% C.I.	a_0	$a_1 \pm$95% C.I.	a_0
Salicylic acid	1.03±0.02	$-2.4\cdot10^{-3}$	0.95±0.03	$2.3\cdot10^{-7}$
2,3-Dihydroxybenzoic acid	1.03±0.02	$-2.9\cdot10^{-3}$	0.94±0.06	$-1.1\cdot10^{-5}$
2,5-Dihydroxybenzoic acid	1.02±0.03	$-3.1\cdot10^{-3}$	0.96±0.02	$9.6\cdot10^{-6}$
Catechol	–	–	0.95±0.02	$7.5\cdot10^{-6}$

The concentration range was 10^{-6}–10^{-4} *M*. Analytical conditions as in Section 2.3.

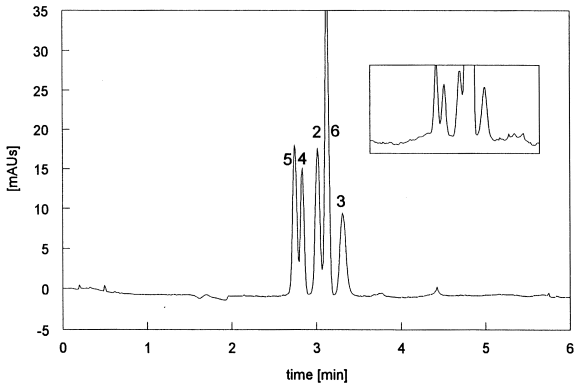

Fig. 4. MECC electropherogram of mixture of salicylic acid (2), 2,3-DHB (3), 2,5-DHB (4), catechol (5) and benzoic acid (6, I.S.). The concentration of each compound was $2\cdot10^{-5}$ *M*. The concentration of the internal standard (I.S.) was $5\cdot10^{-5}$ *M*. Analytical conditions are as described in Section 2.3. The inlay shows an electropherogram of a 10^{-6} *M* mixture of the same composition.

retention order [34,35]. However, we must conclude from comparison of Table 1 Fig. 4 that this is not valid in our case, probably due to the ionic nature of the components. As expected from CZE experiments a wavelength of 200 nm gave the best signal-to-noise ratio. The detection limit, determined as 4-times the signal-to-noise ratio, was also $2\cdot10^{-7}$ *M*. A linear calibration curve was recorded from 10^{-6} *M* to 10^{-4} *M*. The correlation coefficient of all sample components was 0.999. The slope and intercept of the calibration curves for the different sample components are shown in Table 2. The slope (a_1) was determined as dlog(y)/dlog[sample]. For all the samples in the CZE experiments the slope lies between the outer borders of the 95% confidence interval, expecting a slope of 1. The intercept (a_0) was determined from the linear calibration curve.

The calibration curve was calculated without using the internal standard benzoic acid. Contrary to expectation the use of this resulted in a lower correlation coefficient. Comparing this separation with earlier results from Gökören and Tunnel [29] we see that the resolution is slightly lower in our separation. Our detection limit however is a factor $5\cdot10^{3}$ better. Also the time of analysis is a factor of three shorter.

Comparing CZE with MECC it can be seen that the resolution and time of analysis are in the same order of magnitude. The detection limit is the same: $2\cdot10^{-7}$ *M*. Both methods showed good linearity in the range 10^{-6}–10^{-4} *M*. Both analyses did not need an internal standard to improve the reproducibility. Both methods showed fast and accurate analyses. However it has to be noticed that MECC has the advantage that also natural metabolite catechol can be measured in one single run.

4. Conclusions

CZE and MECC both are good analytical separation methods for the determination of salicylic acid and its free radical products 2,3-DHB and 2,5-DHB. With MECC also the other natural metabolite of salicylic, catechol can be determined. The calibration lines were linear over two decades with a detection limit for both MECC and CZE of $2\cdot10^{-7}$ *M*, which would make direct determination in biological samples feasible.

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