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Assay of acetylsalicylic acid and three of its metabolites in human plasma and urine using non-aqueous capillary electrophoresis with reversed electroosmotic flow

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Abstract

The separation of acetylsalicylic acid and three of its metabolites—salicylic acid, salicyluric acid and gentisic acid—is demonstrated in a non-aqueous capillary electrophoresis system with reversed electroosmotic flow. Solvent mixtures of methanol and acetonitrile are used for the electrophoresis media and different electrolytes have been investigated. The flow is reversed by the addition of the polycation hexadimethrine bromide and thus a negative voltage is used. This system provides a fast and effective separation of the four analytes. The separation method was applied to the assay of acetylsalicylic acid and its major metabolites in plasma and urine and the limits of quantification for all of these compounds are about 5 μg ml^{-1} in plasma and 25 μg ml^{-1} in urine.

Keywords: Non-aqueous capillary electrophoresis; Reversed electroosmotic flow; Aspirin

1. Introduction

The separation of anionic solutes may in capillary electrophoresis (CE) lead to extended time of analysis due to their migration in the opposite direction of the electrosmotic flow (EOF). One method to decrease the analysis time is to reverse the electroosmotic flow, thus making the anions migrate in the same direction as the EOF. In aqueous CE the addition of long alkyl chain trimethyl ammonium ions are used for this purpose e.g. in the analysis of inorganic anions [1] and phenols [2]. This principle may also be used in non-aqueous CE. However, the long alkyl chain trimethylammonium ions are not able to form hemimicelles at the inner capillary surface when using non-aqueous solvents and thus the EOF is not reversed. Recently, it has been demonstrated that the addition of low amounts of the polycation hexadimethrine bromide (HDB) to the electrophoresis medium may result in suitable and stable systems with reversed EOF even when using fairly low concentrations (0.001 − 0.05%) in the electrophoresis medium [2,3].

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Acetylsalicylic acid (ASA) has now been used for 100 years and is still one of the most widely used anti-inflammatory drugs. The major metabolites of ASA are salicylic acid (SA), salicyluric acid (SU) and gentisic acid (GA). SA conjugated to glucuronid acid at the carboxylic acid group is also a major elimination product. Furthermore, the phenolic glucuronide of SA, gentisuric acid and 2,3-dihydroxybenzoic acid may be found as metabolites in minor concentrations after administration of ASA [4,5].

As reviewed in [6] numerous analytical methods for the determination of ASA and its metabolites in biological fluids have been published. Only a few methods for capillary electrophoresis of SA [7] and its metabolites [8] in plasma and urine have been reported and neither of these papers describe the simultaneous determination of ASA in biofluids.

In this paper we present a selective, non-aqueous CE method involving migration of the anionic solutes in the same direction as the EOF thus providing a fast assay of ASA and its metabolites SA, SU and GA in human plasma and urine.

2. Experimental

2.1. Chemicals

Acetylsalicylic acid and salicylic acid were of pharmacopoeial quality (Ph.Eur.). Salicyluric acid, hexadimethrine bromide and, β-glucuronidase from Helix Pomatia (100000 U ml^{-1}) were obtained from Sigma (St. Louis, MO). 2,4-dihydroxybenzoic acid (2,4-DHB) and 2,6-dihydroxybenzoic acid (2,6-DHB) were form Fluka AG (Buchs SG, Switzerland). Gentisic acid and all other chemicals being of analytical reagent grade were from Merck (Darmstadt, Germany).

The Oasis® solid phase extraction columns (30 mg) were from Waters (Milford, USA). The solvents and all other chemicals were used as received from the supplier. Aspirin® tablets were from Bayer AG (Leverkusen, Germany).

2.2. Apparatus

An HP^{3D} capillary electrophoresis system (Hewlett-Packard, Walbronn, Germany) equipped with an on-column diode-array detector was used. The following parameters were used for all the analysis unless otherwise stated: The detection wavelength was 214 nm (bandwidth 16). The separations were performed in a fused-silica capillary (64 cm × 50 μm ID; 55.5 cm to the detector) from Polymicro Technologies (Phoenix, AZ). The capillary was thermostated at 25°C with air. Samples were kept at ambient temperature in the autosampler and injected by applying a pressure of 5 kPa (50 mbar) for 3 s. A voltage of -30 kV was applied during analysis.

The electrophoresis medium consisted of methanol and acetonitrile (1:1,v/v) containing 50 mM ammonium acetate and 150 mM sodium acetate and with 0.002% (w/v) HDB added. Prior to use, the capillaries were rinsed with 1 M sodium hydroxide for 60 min, 0.1 M sodium hydroxide for 20 min, distilled water for 20 min and the final electrophoresis medium for 10 min. Between analyses, the capillaries were flushed with electrophoresis medium for 2 min.

2.3. Sample preparation

Plasma sampling was performed in 10 ml vials containing 100 mg of sodium fluoride.

Plasma: 50 μl of internal standard solution (0.5% 2,4-DHB) and 50 μl of concentrated hydrochloric acid were added to 1000 μl of plasma which after centrifugation (3000 g for 10 min) was subjected to solid phase extraction (SPE) on an Oasis® 30 mg column after this had been activated with 1 ml of methanol and then with 1 ml of water. After washing the SPE column with 1 ml of 0.01 M hydrochloric acid the solutes were eluted with 1 ml of methanol. The solvent was evaporated at 25°C using flushing with nitrogen. Finally the residue was dissolved in 1000 μl of methanol.

Urine: 400 μl of internal standard (0.5% 2,4-DHB) and 500 μl of concentrated hydrochloric acid were added to 5000 μl of urine and the mixture was diluted to 10.00 ml with water. A

total of 1000 μl of this solution was subjected to SPE as described under sample preparation of plasma.

When the urine was treated with enzymes 1 ml of 0.1 M sodium acetate pH 5.0 and 50 μl of, β-glucuronidase were added to 5000 μl of urine. After 24 h at 37°C the mixture was treated as above.

Table 1
R_s between neighbouring solutes including hippuric acid (HA) in the electropherogram as a function of the temperature

Solute pair	15°C	25°C	35°C	45°C	55°C
ASA-HA	5.09	3.85	2.59	1.45	<0.6
SU-ASA	1.59	2.59	3.82	4.91	5.91
IS-SU	8.17	7.52	6.77	6.23	5.59
GA-IS	4.41	4.35	4.30	4.34	4.38
SA-GA	11.6	11.2	10.6	10.4	9.77

3. Results and discussion

The centenary anniversary for the analgesic, anti-inflammatory and antipyretic drug substance acetylsalicylic acid (ASA) has recently been celebrated and the drug substance is still widely used. The development of new and simplified analysis methods for the assay of ASA and it major metabolites in biological samples is therefore still of interest. The high selectivity of separation obtained in non-aqueous CE systems [9,10] combined with reversed EOF should therefore provide a fast and efficient separation system for this purpose.

3.1. The electroosmotic flow

ASA and its major metabolites are all anionic in nature at neutral pH and the solutes will therefore migrate in the opposite direction of EOF and thus result in a longer analysis time.

Recently, it has been shown that HDB is useful for altering the charge on the fused silica surface. When the charge is changed from negative to positive the EOF is reversed and EOF will be in the direction away from the detector. Therefore, also the polarity was reversed. The use of HDB with a concentration of 0.001% resulted in CE systems that were not sufficient stable with respect to migration times. The concentration was therefore raised to 0.002% which gave only a little increase in EOF but provided repeatable migration times.

3.2. The organic solvent

In order to be able to perform detection at wavelengths in the low UV region methanol and/or acetonitrile are the most obvious choices as solvents for non-aqueous CE. When using neat methanol EOF is decreased resulting in long analysis time, and when using neat acetonitrile limited solubility of the electrolytes is observed. Therefore, a mixture of methanol and acetonitrile was used for further experiments. In 75% acetonitrile sodium acetate still has a limited and insufficient solubility. When using 25% acetonitrile ASA and SU comigrated. A mixture of equal volumes of methanol and acetonitrile was therefore used in the experiments.

Table 2
Accuracy and repeatability expressed as % recovery and coefficient of variation (CV) ($n = 6$) for ASA, its metabolites and two internal standards in plasma

Concentration added (mg ml^{-1})	2,6-DHB	SA	GA	2,4-DHB	SU	ASA
10	66 ± 4.0	85 ± 3.0	76 ± 5.0	90 ± 3.2	94 ± 3.0	99 ± 3.1
50	48 ± 3.1	86 ± 1.8	77 ± 2.8	89 ± 0.8	93 ± 0.8	92 ± 1.1
200	25 ± 3.4	93 ± 3.0	65 ± 4.5	85 ± 2.7	98 ± 2.4	96 ± 1.3

Table 3
Accuracy and repeatability expressed as % recovery and coefficient of variation (CV) ($n = 6$) for ASA, its metabolites and two internal standards in urine

Concentration added (mg ml^{-1})	2,6-DHB	SA	GA	2,4-DHB	SU	ASA
50	92 ± 3.4	97 ± 3.5	99 ± 3.7	95 ± 3.5	88 ± 2.7	87 ± 3.3
200	94 ± 2.2	91 ± 1.0	90 ± 1.7	88 ± 1.7	87 ± 2.2	84 ± 2.4
500	81 ± 4.6	95 ± 2.8	75 ± 5.4	88 ± 3.8	92 ± 3.7	87 ± 2.9

3.3. pH* and nature of the electrolyte

The dependence of changes in pH* and of changes in the nature and concentration of the electrolyte on the separation selectivity was investigated. When using ammonium acetate as the only electrolyte hippuric acid (HA) being a major constituent of urine interfered with the determination of ASA. If increasing amounts of sodium acetate were added to the electrophoresis medium increasing selectivity between the analytes and the remaining anionic interferences from the urine was obtained. The analytes had a higher migration rate towards the anode (at the detection end of the capillary) which probably is due to the phenolic groups on the analytes contributing to an increase in negative charge when the pH* is raised (8–9.5).

3.4. The temperature

The influence of the temperature surrounding the capillary on the selectivity of the separation was investigated in the temperature range 15–55°C. Table 1 shows the resolution R_s determined for neighbouring solute pairs as a function of the temperature.

$$R_s = \frac{1.17(t_{R_1} - t_{R_2})}{(W_{h_1} + W_{h_2})}$$

At lower temperatures ASA and SU migrate very close to each other and at higher temperatures ASA and HA comigrate. Therefore a temperature of 25°C was chosen.

4. Validation

A preliminary validation of the method developed was performed. The response of all the analytes and the internal standard was found to be linear in the range 5–500 μg ml^{-1}. The 5 μg ml^{-1} was also the limit of quantification (determination with a coefficient of variation (CV) $< 20\%$) in plasma. In urine the limit of quantification was higher (25 μg ml^{-1}) due to small amounts of interfering, endogenous substances.

Accuracy and repeatability were investigated using standard addition of known amounts of the analytes to blank plasma and urine, respectively. For the determination of the recovery, the samples were compared with calibration standards which were not treated by SPE.

Tables 2 and 3 gives the recoveries of each of the analytes at three concentration levels in plasma and urine, respectively. The repeatability is expressed as the CV for $n = 6$.

Good recoveries are obtained for most analytes. Only 2,6-DHB shows a low recovery. This substance was considered for the use as internal standard but it has also a migration that differs somewhat from the analytes and it was therefore rejected as internal standard. 2,4-DHB had a suitable migration and a good recovery and was therefore chosen as the internal standard.

The repeatability for all the analytes is good.

5. Application

The applicability of the method developed is illustrated by the analysis of plasma and urine samples collected from a volunteer (male 50 years old) having taken 1.5 g of ASA p.o. as a single dose. Fig. 1 shows the electropherogram of a plasma sample and a urine sample 1 and 2 h after dosage, respectively. Electropherograms of the

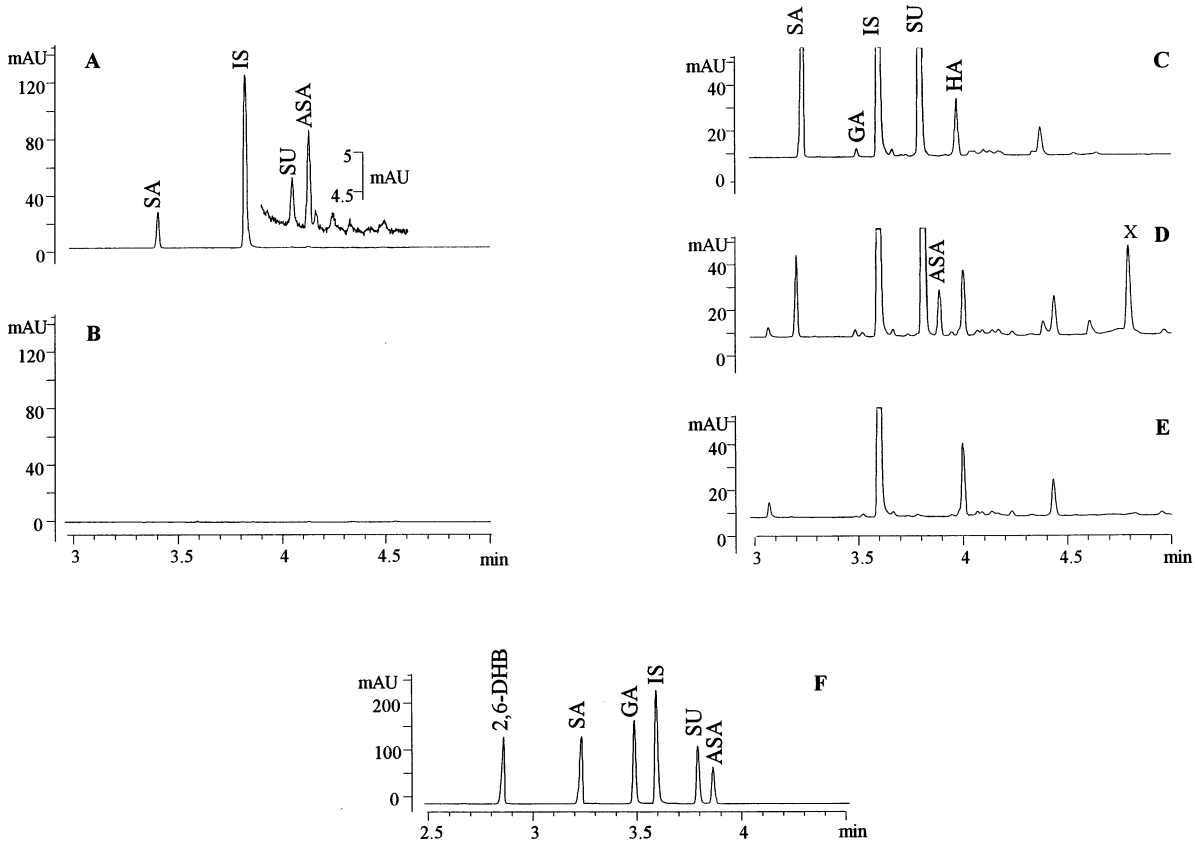

Fig. 1. Electropherograms of (A) plasma sample 1 h after administration of 1.5 g ASA p.o. (35, μg SA ml^{-1} and 2.7 μg ASA ml^{-1}); (B) plasma blank; (C) enzyme treated urine sample 0–2 h after administration of 1.5 g ASA p.o.; (D) the same urine sample without enzyme treatment; (E) blank urine (with IS added) and (F) calibration standard (200 μg ml^{-1} of each solute). Electrophoresis: fused-silica capillary (64 cm × 50, μm ID; 55.5 cm to detector); detection, 214 nm; temperature 25°C; voltage, −30 kV. Electrophoresis medium: 50 mM ammonium acetate and 150 mM sodium acetate in methanol and acetonitrile (1: 1, v/v) with 0.002% of HDB added. ASA, acetylsalicylic acid; SA, salicylic acid; GA, gentisic acid; SU, salicyluric acid; HA, hippuric acid; 2,6-DHB, 2,6-dihydroxybenzoic acid; IS, internal standard = 2,4-dihydroxybenzoic acid. X, a glucuronide of SA.

corresponding blank urine as well as blank plasma are also displayed.

6. Conclusion

A non-aqueous capillary electrophoresis system for the assay of acetylsalicylic acid and its major phase I metabolites in plasma and urine has been developed. The method involves solid phase extraction followed by the separation where the solutes are detected by UV absorbance at 214 nm. The non-aqueous CE system provides high selectivity and the reversed EOF also reduces the analysis time for the anionic solutes to < 5 min.

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