ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
17 (1998) 1161–1166

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Determination of phenprocoumon in plasma and urine using at-line solid-phase extraction-capillary electrophoresis

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Received 21 February 1998; accepted 23 February 1998

Abstract

The use of capillary electrophoresis (CE) for the analysis of biological samples is rather problematic because of the large number of interferences present in the matrix. One of the possibilities to solve such problems is to couple solid-phase extraction (SPE) at-line with CE, a technique developed in our laboratory. In this study at-line SPE–CE is performed for the determination of the anticoagulant phenprocoumon in biological fluids. Plasma samples are injected after the addition of 1 vol.% of formic acid to release the drug from binding proteins, while urine samples can be directly injected. The procedure is linear between 0.2 and 30 μg ml^{-1} with a correlation coefficient, r^2, of 0.9996. The detection limit in plasma is 0.1 μg ml^{-1}, which is fully adequate in view of the concentrations, that have to be dealt with in practice. The phenprocoumon concentration in a plasma sample of a patient treated with the anticoagulant was 3.8 μg ml^{-1}.

Keywords: At-line; Capillary electrophoresis; Coumarines; Phenprocoumon; Sample preparation; Plasma; Solid-phase extraction; Urine

1. Introduction

Capillary electrophoresis (CE) is rapidly gaining acceptance for routine analyses even though sample handling in, e.g. bioanalyses still creates problems. Biological samples frequently contain high concentrations of organic and inorganic salts, proteins, particulate matter and many low molecular-weight compounds. Moreover, the concentrations of the analytes of interest are usually low. Various approaches can be used for the clean-up of a sample and trace enrichment of the analytes, which are based on dialysis, chromatographic and/or electrophoretic principles. In this study, solid-phase extraction (SPE) coupled at-line to CE was the preferred option. To this end, an ASPEC sampling-handling device was used [1]. A fresh (disposable) cartridge was used for every run to avoid carry-over effects. The present study deals with the determination of the anticoagulant phenprocoumon (for the chemical structure see Fig. 2), after oral administration, in plasma and

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0731-7085/98/$19.00
PII S0731-7085(98)00082-X

urine. The total concentration of phenprocoumon in the plasma of patients, who typically use a daily dose of 1.5–7.5 mg, ranges from 0.3 to 5.1 μg ml^{-1} [2]. Its therapeutic window in human plasma is between 0.2 and 5 μg ml^{-1}; at concentrations >5 μg ml^{-1} toxic effects are observed. As a result of a rapid metabolism in the body, the parent compound is normally not found in urine [2]. The aim of the present study is to demonstrate the advantages of a fully automated procedure over current off-line procedures such as those reported in [2–4].

2. Experimental

2.1. Chemicals and samples

Methanol, acetic acid, phosphoric acid, formic acid and acetonitrile were obtained from J.T. Baker (Deventer, The Netherlands). All chemicals used were of HPLC quality. Water was demineralized and distilled before use. Phenprocoumon was a gift of Roche Nederland (Mijdrecht, The Netherlands).

Urine was collected during three consecutive days from five healthy male volunteers, pooled, and frozen in small quantities at -18°C. The plasma of healthy volunteers was a gift from the Academic Hospital of the Free University and was stored at -18°C. The patient was treated orally with 5.0 mg of phenprocoumon a day and the plasma collected in heparinized tubes. The biological samples were stored for a maximum of 6 months at a temperature of -18°C.

An acetonitrile/12.5 mM phosphate buffer of pH 4.5 (20/80, V/V) was used as SPE wash buffer. The desorption buffer consisted of acetonitrile/40 mM phosphate buffer of pH 7.8 (75/25, V/V). A 20 mM acetate buffer, pH 4.6 /methanol (90/10, V/V) solution was used as CE buffer.

2.2. SPE equipment and procedures

SPE experiments were performed fully automated using an ASPEC system (Gilson, Villiersle-Bel, France). LC-18 disposable cartridges (Supelco, Bellefonte, PA) packed with 100 mg C-18 bonded silica material were used. The cartridges were washed with, subsequently, 2 ml of methanol and 2 ml of 10 mM phosphoric acid at a flow of 3 ml min^{-1}, prior the sample loading. In the case of urine, 8 ml of sample were loaded without any pretreatment (at flow of 3 ml min^{-1}). For plasma samples, 1 ml was loaded at 1 ml min^{-1}. Before loading, the plasma samples were acidified with 1 vol.% of formic acid and after vortex mixing (1 min) the interferences were removed with 3 ml of wash buffer purged through the SPE column with 1 ml of air. For plasma samples the SPE cartridge was also flushed with 1 ml of 10 mM phosphoric acid solution before and after the washing procedure. For both urine and plasma, the analytes were desorbed with 400 μl of desorption buffer at 400 μl min^{-1}.

2.3. Interfacing SPE and CE

After completion of the SPE procedure, the ASPEC injected a volume of 190 μl out of 400 μl of desorption fluid into a 100 μl loop, constructed of PEEK tubing (0.25 mm i.d.), see Fig. 1. After switching the six-port valve, the loop content was flushed to the interface, which was essentially the same as given for the at-line SPE-CE as described in an earlier paper [1]. The interface was constructed of 7 mm of green PEEK tubing (0.75 mm i.d., 1/16 in. o.d.) and 30 cm of red PEEK tubing (0.13 mm i.d., 1/16 in. o.d.), which were placed inside a piece of PTFE tubing with a length of 37 mm (1/16 in. i.d., 1/8 in. o.d.); details of the positioning can be seen in Fig. 1. One end of the red PEEK tubing was inserted over a length of 18 mm in the PTFE tubing; the other end was connected to the valve. The CE capillary was inserted into the green PEEK tubing and the electrode was placed on top of this tubing, (see insert of Fig. 1). Since, only a minor part of the contents of the loop can be injected into the CE capillary. Injection was performed during the period that the plug containing the highest concentration of analytes passed the tip of the capillary, which is between 0.2 and 0.5 min after switching of the 6-port valve. During the injection an under pressure of 40 mbar at the other end of the CE capillary was applied. The PrinCE and the

ASPEC were electronically connected in order to synchronise the sample preparation, injection and CE procedures.

2.4. CE set-up

A PrinCE (Prince Technologies, Deventer, The Netherlands) CE system was used. The CE capillary (50 μm i.d., 375 μm o.d.), with a total length of 96 cm and an effective length of 40 cm (LC-Service, Emmen, The Netherlands). The CE system was equipped with a thermostating device set at 20°C by blowing air around the capillary (35 m s^{-1}) [5]. The capillary was conditioned by flushing it successively with, 1 M aqueous sodium hydroxide (5 min), water (15 min) and CE buffer (30 min); in all cases a pressure of 2000 mbar was used.

Prior to each injection, the capillary was rinsed for 2 min with CE buffer. Analyses were performed using a voltage of −30 kV (the electrode in the interface was grounded) and detection was performed using a model 759A UV/VIS absorbance detector, (Applied Biosystems, Foster City, CA), at 200 nm.

3. Results and discussion

3.1. Analysis of plasma samples

For the determination of the total phenprocoumon concentration in plasma 1 vol.% of formic acid has to be added to disrupt the drug-protein binding [1]. After vortex mixing, the SPE-CE procedure was used to determine phenprocoumon

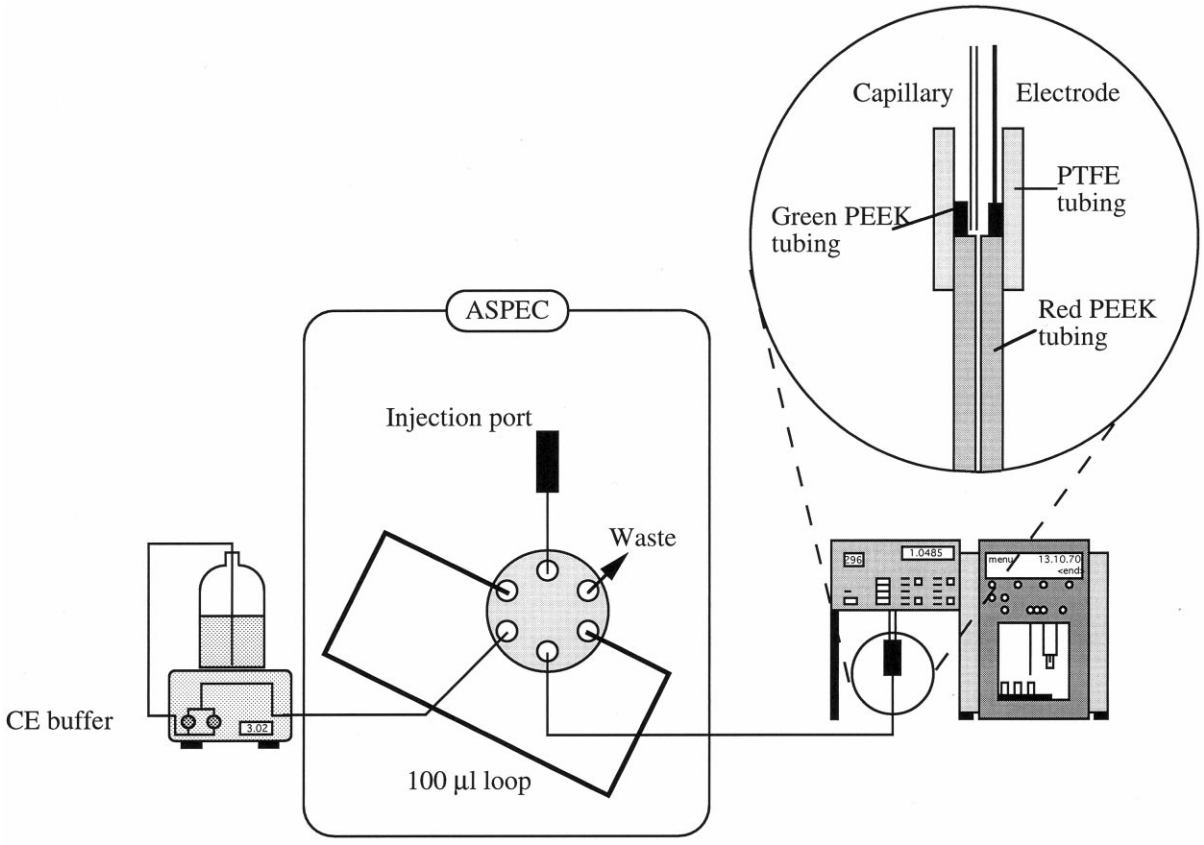

Fig. 1. Schematic representation of the SPE–CE set-up.

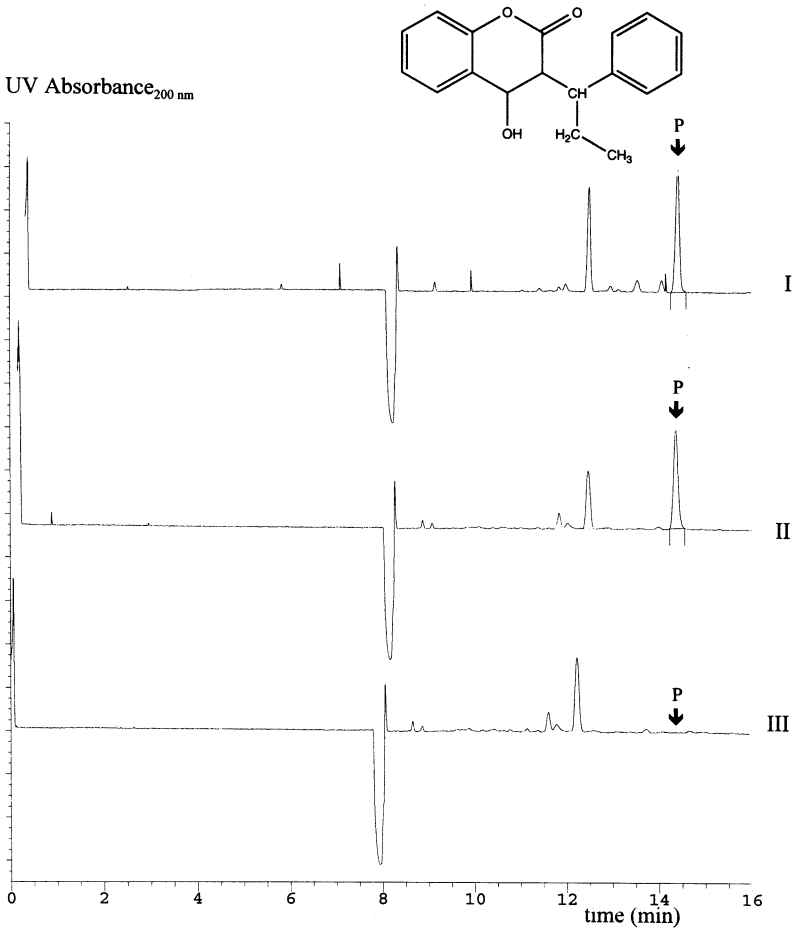

Fig. 2. At-line SPE–CE electropherograms of plasma samples after addition of 1 vol.% of formic acid of plasma from (I) a patient treated with 5.0 mg phenprocoumon a day, (II) a healthy volunteer, spiked at 3 μg ml^{-1} and (III) a healthy volunteer.

in the plasma of a healthy volunteer (blank and spiked with 3 μg ml^{-1} phenprocoumon) and of a patient treated orally with 5.0 mg of phenprocoumon a day. The electropherograms of Fig. 2 show a fully satisfactory separation of phenprocoumon from their sample constituents (traces I and II), and the absence of interfering compounds in the blank sample (trace III). The calibration curves were constructed in the range of 0.2–30 μg ml^{-1}, using eight data points ($n = 2$). Linearity was good with a slope of 0.386 (SD = 0.003), an intercept of -0.01 (SD = 0.04) and correlation coefficient, r^2, of 0.9996. The linear dynamic range covers both the therapeutic range of phenprocoumon, which is between 0.25 and 5 μg ml^{-1} [2], and the toxic range, which is between 5 and 20 μg l^{-1} [2]. The detection limit of 0.1 μg ml^{-1} (signal to noise ratio, 3/1) is sufficiently low to enable the determination of phenprocoumon in plasma under real life conditions. In the plasma of the patient treated with the drug, the phenprocoumon concentration was found to be 3.8 μg ml^{-1}. This results with values reported in the literature [2].

3.2. Analysis of urine samples

No sample pretreatment was necessary for the

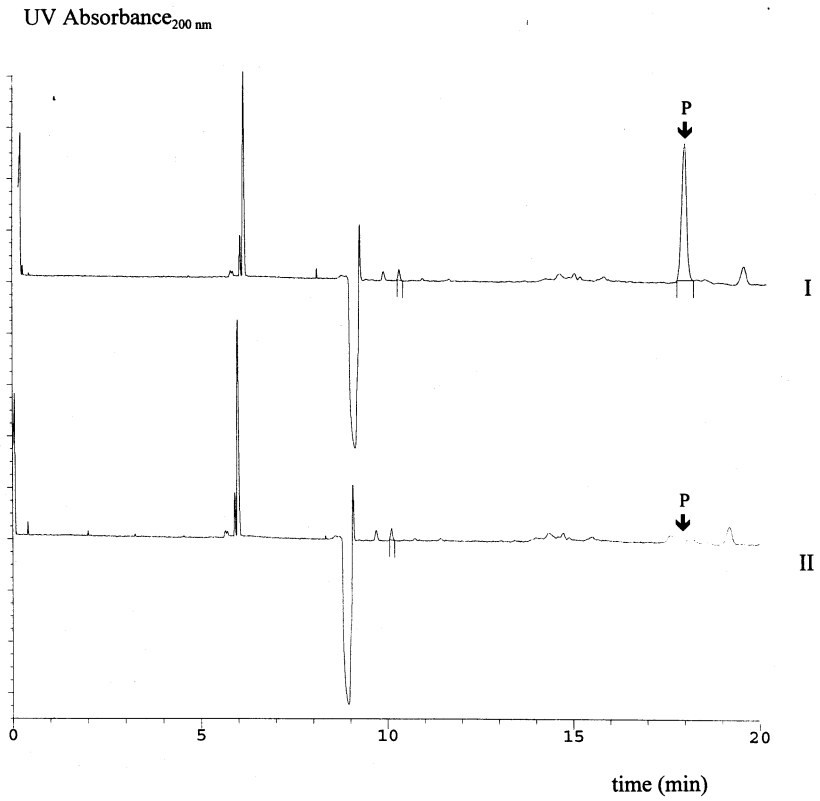

Fig. 3. At-line SPE–CE electropherograms of urine samples from a patient treated with 5.0 mg phenprocoumon a day; (I) spiked to a level of 3 μg ml^{-1} and (II) non-spiked urine.

analysis of urine samples; they could be directly applied to the SPE cartridge. The electropherograms of Fig. 3 shows that the trace level detection of phenprocoumon is straightforward. Due to the fact that larger samples can be processed (1 ml for plasma, 8 ml for urine) the detection limit is consequently better, i.e. 0.02 μg ml^{-1}. It is also obvious from Fig. 3, that there is no phenprocoumon in the urine of the treated patient, even though the plasma contained ca 4 μg ml^{-1}. This can be explained by the fact that the drug is completely metabolized. It is known to be hydroxylated, the main metabolites being 4-, 6- and 7-hydroxyphenprocoumon [4]. Unfortunately, as far as we know these compounds are not commercially available. Consequently, no attempt could be made to establish their possible presence.

4. Conclusions

It has been shown that the fully automated at-line SPE–CE procedure can be applied for the quantitative determination of the anticoagulant phenprocoumon in plasma and urine. After the samples were placed in the ASPEC, the SPE procedure, transfer to the CE system and the CE analysis were performed automatically. This in contrast with procedures presented in the literature which includes several manual sample

preparation steps. The sample throughput is two samples per hour. A capillary was used for the analyses of 250 samples before replacement. It was flushed after 50 samples sodium hydroxide solution. The SPE cartridges were used once for biological samples to avoid carry-over effects. No blocking of the loop, interface or capillary was found, and therefore, the system was successfully used for overnight experiments.

Plasma samples required the addition of formic acid, to disrupt the drug-protein binding, while urine could be analysed directly. The calibration curve and the detection limits in serum samples are fully adequate to cover the relevant toxic and therapeutic ranges. The concentration of phenprocoumon found in the plasma of a patient, and its absence in urine is in agreement with the literature data.

Acknowledgements

The authors thank Roche Nederland B.V. and the Academic Hospital of the Vrije Universiteit, for their gifts of phenprocoumon and plasma, respectively.

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