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Quantitation of acetaminophen and salicylic acid in plasma using capillary electrophoresis without sample pretreatment Improvement of precision

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Abstract

Capillary electrophoresis has become one of the most attractive techniques in the analysis of biological samples. Pharmaceuticals in human plasma can easily be determined on uncoated fused-silica capillaries without any sample pretreatment. Intra- and inter-day precision values of about 1–2% R.S.D. ($n=20$) and 2–3% R.S.D. ($n>80$) respectively are obtained using a sodium dodecyl sulfate-containing borate buffer, pH 10 and acetonitrile as a between-run rinsing reagent. This method is highly robust, no breakdowns of the current or capillary blockings were observed for several weeks. The general applicability is demonstrated for several model drugs. The effectiveness of other rinsing procedures including enzyme-containing solutions, different organic solvents and hydrofluoric acid is discussed.

Keywords: Direct injection; Rinsing procedures; Acetaminophen; Salicylic acid

1. Introduction

Capillary electrophoresis (CE) offers great advantages in the analysis of biological material [1]. Most of the common techniques applied in clinical chemistry inevitably require sample pretreatment steps (liquid–liquid or solid-phase extraction). This is not necessary when using CE analysis. Body fluids like urine, serum, or plasma can be injected directly onto the capillary after just a simple filtration step to remove particles which would block the capillary [2–4]. This option provides easy, time and sample saving, error reduced analysis.

Using the micellar electrokinetic chromatography mode with sodium dodecyl sulfate (SDS) as the micelle-forming agent, matrix proteins associate to negatively charged aggregates. Due to their high charge/mass ratio, these complexes have got a rather high electrophoretic mobility towards the anode, but in consequence of the electroosmotic flow (EOF), which is directed towards the cathode, a low gross mobility to the latter. Thus, they move rather slowly leaving an "analytical window" in which drug molecules can be detected [5,6]. As a further advantage, SDS completely releases the plasma protein binding of drugs [3]. Therefore, drug levels determined are total concentrations, data which are typically employed in drug monitoring and pharmacological research.

However, the complex matrix of biological samples causes a problem which needs to be overcome. Matrix proteins tend to adhere to the capillary wall due to interactions between negatively charged silanol groups and positively charged moieties of the protein molecules like arginine and lysine residues [7,8]. This causes a decrease in the EOF resulting in

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peak broadening and loss of efficiency and reproducibility [9]. Since adsorption can be very strong for some protein fractions (e.g. albumin) due to thermodynamic gains [10], an effective rinsing reagent is required to remove the adsorbed plaques. It was shown that SDS alone was not sufficient for this purpose. This was shown elsewhere by scanning electron microscopy of the inner capillary surface [11]. Thus, for a reliable quantitation it is essential to find an appropriate rinsing reagent. The present work discusses the effect of different rinsing procedures and presents a generally applicable, reliable and reproducible method for the quantitation of drugs in plasma with direct sample injection.

2. Experimental

2.1. Apparatus

CE separations were carried out using a SpectraPhoresis 1000 system by Thermo Separation Products (TSP, Fremont, CA, USA) with PC 1000 Software 3.0.1 controlled by a personal computer featuring OS/2 Warp operating system. Integration was done by a laboratory built integration program (K.I.S.S., Würzburg, Germany). Fused-silica capillaries from Polymicro Technologies (Phoenix, AZ, USA) were used in all experiments. The typical capillary dimensions were 50 μm inner diameter (375 μm outer diameter), 42.5 cm total length (L) and 34.5 cm from the inlet to the detector window (l, effective length). The capillary was assembled in the PC 1000 cartridge. On-line UV detection was performed at 200 nm with a rise time of 1.0 s, if not otherwise stated. The capillary was maintained at 25°C with forced air circulating in the capillary oven. The high voltage power supply was set to 10 kV (normal polarity, equivalent to a field strength of 235 V/cm) resulting in a typical current of 42–44 μA. The detection window was at the cathode side. Injections were made at the anode side by applying a vacuum (0.75 p.s.i.≅50 mbar) for 3–4 s. Before the first use the capillary was conditioned with freshly prepared 0.1 M NaOH for 30 min at 50°C and then equilibrated with cathode buffer for 2 h at 25°C applying the separation voltage of 10 kV. Between-run washings are mentioned below. When long automated sequences of samples were being run, different wash and run buffer vials were used to avoid lowering of the fluid level in the run buffer vial and consequent hydrodynamic siphoning [12]. Additional experiments were carried out using a Crystal 310 CE system by ATI Unicam (Madison, WI, USA) equipped with a variable-wavelength detector. The CE system was controlled by a MS-DOS personal computer featuring MS Windows for Workgroups 3.11 with Crystal CE Control Software 1.3β. Data acquisition was done with Unicam 4880 Chromatography Data Handling System 1.00. Fused-silica capillaries as described above were used with a total length of 60 cm and an effective length of 45 cm. The capillary was maintained at ambient temperature (25°C). On-line UV detection was done at 200 nm with a rise time of 1.0 s if not stated otherwise. Samples were introduced at the anode side applying a pressure of 50 mbar for 0.1 min. Separation voltage was 15 kV (normal polarity, equivalent to a field strength of 250 V/cm) corresponding to a current of 50 μA. Conditioning and equilibration of new capillaries was performed in the same way as described above.

2.2. Chemicals

Boric acid, sodium hydroxide, sodium chloride, potassium nitrate and hydrochloric acid were of analytical grade, sodium salicylate and hydrofluoric acid of laboratory grade, SDS was of microbiological grade, methanol and acetonitrile were of chromatography grade. All were obtained from Merck (Darmstadt, Germany). Trypsin and chymotrypsin (microbiological grade) were purchased from Sigma (Deisenhofen, Germany). Acetaminophen (pure) was obtained from Fluka (Neu-Ulm, Germany), other drugs were of German pharmacopoeia grade (donations by different German pharmaceutical companies). Water was of high-performance liquid chromatography-quality prepared on a Milli-Q station (Millipore, Eschborn, Germany). All solutions were filtered through 0.2 μm membrane filters (Schleicher and Schuell, Hartenstein, Würzburg, Germany). Human plasma was obtained from the Red Cross (Würzburg, Germany).

2.3. Buffer and sample preparation

The electrophoretic separations were performed using a borate buffer with SDS as surfactant additive. The cathode buffer was a 60 m*M* borate buffer, pH 10.0. Therefore 3.170 g of boric acid were dissolved in 100 ml of water, adjusted to a pH of 10.0 with 1 *M* NaOH solution (freshly prepared) and made up with water to 1000.0 ml. It was used for the first equilibration of the capillary and served for filling the cathode reservoir. The running buffer was prepared by making up 5.768 g of SDS to 100.0 ml with cathode buffer to give a final concentration of 200 m*M*.

Human plasma containing stabilizer (citrate, dextrose and phosphate) was kept frozen at −20 or −40°C and was allowed to thaw at room temperature without using heat or ultrasonication. The plasma used was 6 to 10 months old (according to German regulations, plasma used for medical treatment has to be stored below −30°C and may then be used up to 12 months). Before use plasma was filtered through a 0.2 μm membrane filter. Stock solutions of the drugs were prepared by making up 100 mg of each compound to 100.0 ml with physiological sodium chloride solution (9 g/l) and were filtered before use. To obtain the sample solution, 100 μl of stock solution were spiked to 900 μl of plasma and gently shaken for 5 min to give a final concentration of 0.1 g/l.

Enzyme solutions were prepared by making up 20 mg of trypsin or chymotrypsin to 5.0 ml with hydrogenphthalate buffer pH 4.00 for trypsin and phosphate buffer pH 7.43 for chymotrypsin respectively. During enzyme washes the capillary oven temperature was set to 37°C. Hydrofluoric acid etching of the capillary was performed off-line using a Vacuubrand vacuum pump (Brand, Wertheim, Germany). Hydrofluoric acid (10%) was flushed through the capillary for 30 s followed by 0.1 *M* HCl for 3 or 5 min and water for 3 or 5 min.

3. Results and discussion

A borate buffer with a high pH was chosen because high concentrations of macromolecules, especially proteins, were present in the samples to be analyzed. At a pH of 10 the majority of the silanol groups are negatively charged as well as the plasma proteins (isoelectric point, p*I*<7). Hence, interactions between sample components and the wall should be reduced. Additionally, the ionic strength of the buffer was rather high. This is beneficial for separation efficiency but at the same time limits the possibility of applying higher voltages. Thus, a moderate value of 10 kV was chosen resulting in an adequate current of about 40 μA. The use of SDS as micelle-forming agent to wrap up the proteins implies two advantages. First of all, the protein–SDS aggregates are negatively charged and interaction with the negatively charged wall is reduced. Moreover, these complexes move slowly towards the cathode due to their high charge/mass ratio and leave an "analytical window" in which drug molecules can be detected. Fig. 1 shows the large fraction of matrix proteins between 20 and 40 min. Pharmaceutical compounds can be detected in the window between 0 and 20 min. Note that for the cathode reservoir the plain borate buffer (cathode buffer) was used to avoid the formation of tenside bubbles which might creep up to the detector window at the outside of the capillary and consequently might deteriorate the detection. This holds true for instruments with automatic buffer replenishment. The electropherogram also illustrates impressively the dimensions of the ratio between matrix and analytes. From this it becomes clear that washing out proteins between runs is a quite challenging, but indispensable task in order to reach stable conditions and reliable quantitation.

For this purpose different approaches were made. The tested rinsing reagents are summarized in Table 1. A stable, reproducible separation was only achieved with acetonitrile and SDS-buffer. A mixture of 50% acetonitrile and 50% SDS-buffer proved to be best compared to a higher acetonitrile concentration or pure acetonitrile. After each run the capillary was first cleaned with the above mentioned mixture for 15 min and was then re-equilibrated with running buffer for another 20 min. The results obtained with this method are shown in Table 2. For both acetaminophen and salicylate reproducibilities of relative peak areas were good, especially for intra-day series (compare Fig. 2). A deterioration in reproducibility occurs when the analyte overlaps

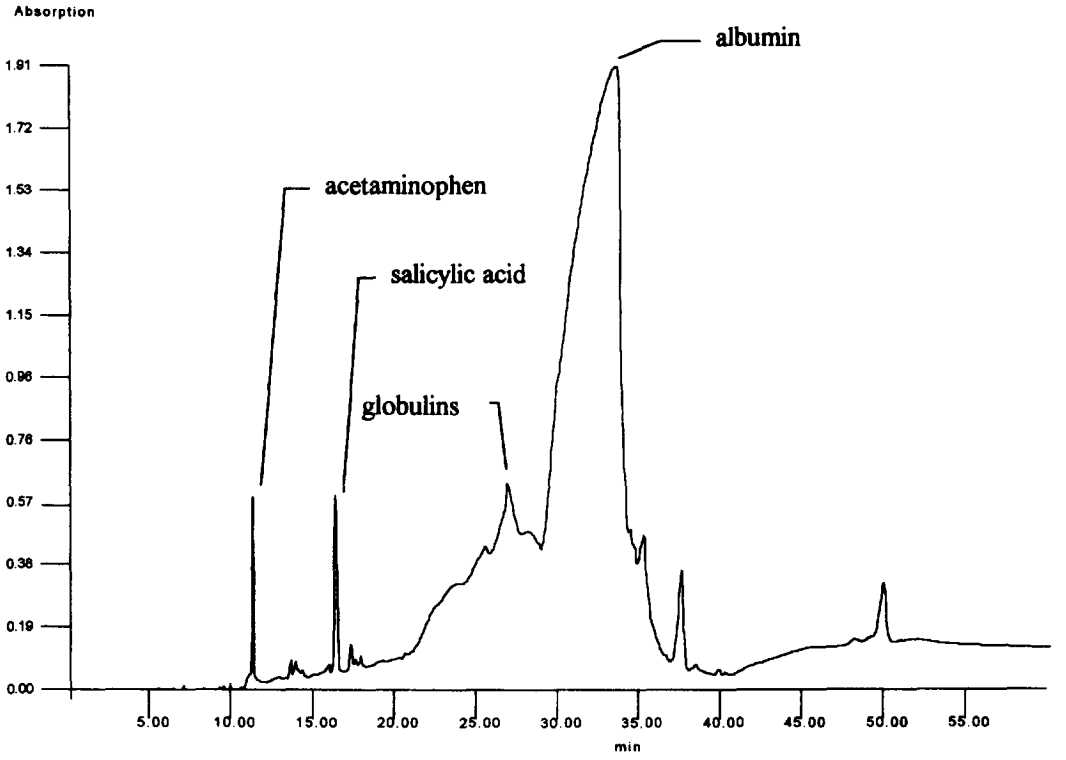

Fig. 1. Human plasma spiked with acetaminophen ($t_M \cong 11$ min) and salicylate ($t_M \cong 16$ min), 0.1 mg/ml each. Separation conditions: borate buffer 60 m*M*, pH 10.0 containing 200 m*M* SDS; injection time 4.0 s (0.75 p.s.i.), 10 kV (42 μA), L=42.5 cm, l=34.5 cm, λ=200 nm; separation was performed on the TSP instrument; further details are given in Section 2. The separation shows the large fraction of matrix proteins between 20 and 40 min with the globulins migrating in front of albumin (compare [14]). In the "analytical window" between 0 and 20 min drug molecules can be detected.

Table 1
Rinsing reagents tested for between-run washes

Reagent	Concentration/procedure	Remarks
NaOH	0.1 mol/l	No stable separation Fluctuating current,
Methanol–SDS-buffer	40% (v/v) methanol, 235 m*M* SDS	Current breakdowns
Salt solutions	NaCl KNO_3 (both saturated in water)	No peaks distinguishable No stable current
Enzymes	Trypsin (pH 4.0) Chymotrypsin (pH 7.5)	Stable current Very low separation efficiency, broad peaks
Hydrofluoric acid (HF)	Etching with HF (10%) followed by 0.1 *M* HCl and water (carried out twice)	Stable current Peak broadening after about 15 runs
Acetonitrile–SDS-buffer	50% (v/v) acetonitrile, 100 m*M* SDS	Stable current Stable, reproducible separation

Table 2
Reproducibility data for direct injection of plasma spiked with acetaminophen and salicylate

	Concentration (g/l)	t_M (R.S.D.%)	Rel. area (R.S.D.%)
Intra-day: $n=20$			
Acetaminophen	0.1	0.73	2.32
	0.01[a]	1.27	4.46
Salicylate[b]	0.086	1.73	1.01
	0.043[a]	2.78	2.57
Inter-day: $n=42/85$[c]			
Acetaminophen[d]	0.1	5.75/4.65	4.84/5.02
Salicylate[b]	0.086	5.37/5.06	2.16/2.24

The separation buffer was a 60 m*M* borate buffer, pH 10.0 containing 200 m*M* SDS. Separation conditions: injection time 3.0 s (for intra-day data) or 4.0 s (for inter-day data), hydrodynamically (0.75 p.s.i.≅50 mbar), 10 kV (42–44 μA); capillary: $L=42.5$ cm, $l=34.5$ cm, I.D.=50 μm; $\lambda=200$ nm, if not stated otherwise; for further information, see Section 2.
[a]detection wavelengths $\lambda=254$ nm for acetaminophen and $\lambda=275$ for salicylate; capillary length was $L=28$ cm, $l=20$ cm; voltage was 8 kV.
[b]concentration calculated for salicylic acid.
[c]data of two independently recorded sets.
[d]for the interpretation of acetaminophen data, see Section 3 and Fig. 3.

with minor plasma components. This is the case with acetaminophen. Fig. 3 illustrates the data of salicylate which represents the performance of the method which is good even for inter-day series. In contrast to that, data of acetaminophen show the performance of the system obtained under less favourable conditions, i.e., when interferences between endogenous matrix components and the analyte appear. In this case the reproducibility could be improved by changing the selectivity, e.g., by slightly modifying the buffer pH or the temperature. Another possibility is to choose a more selective, i.e., a higher wavelength. If the capillary is stored overnight, it is recommended to rinse it with buffer applying a small voltage to avoid ageing of the adsorbed proteins. This would diminish the effectiveness of the rinsing regimen.

Other approaches were not suitable. Salt solutions (NaCl, KNO_3) did not help to overcome the problem described above, because no separation was achieved and the current was unstable in subsequent runs. Highly concentrated enzyme solutions were subsequently flushed through the capillary for 10 min and were then allowed to react for another 5 min (first trypsin at pH 4, then chymotrypsin at pH 7.5). After that the capillary was rinsed with running buffer. This procedure did not permit the components to be resolved, and very broad peaks were observed, probably due to adsorption of the enzyme itself to the capillary wall (even additional rinsing with acetonitrile–SDS-buffer did not change the separation pattern). Solutions of 0.1 *M* NaOH and methanol-containing SDS-buffer are principally suitable, but still current irregularities occurred and thus, outliers during sample series were observed. Especially when NaOH was used, these outliers were characterized by increasing current during a run, current breakdowns or fluctuations. Furthermore, the baseline in the analytical window was raised in some cases. This probably results from the desorption of protein fragments from the wall during runs. Etching of the capillary by hydrofluoric acid treatment was supposed to smoothen the capillary surface and remove surface cracks and defects (compare [11]). This should reduce the inner capillary surface and thus protein adsorption. In fact, the opposite was observed.

A stable and reproducible separation could only be achieved using a 1:1 mixture of acetonitrile and SDS-buffer as rinsing reagent. Fig. 4 demonstrates the applicability of the method to other drugs, e.g., sulfamethoxazole and tolbutamide, which migrate within the "analytical window" (electropherogram of trimethoprim not shown: migration time, $t_M=16.5$ min, ATI Unicam instrument, further conditions as given in Section 2).

In CE reproducibility strongly depends on sample concentrations. High sample concentrations were used to avoid additional error sources in order to

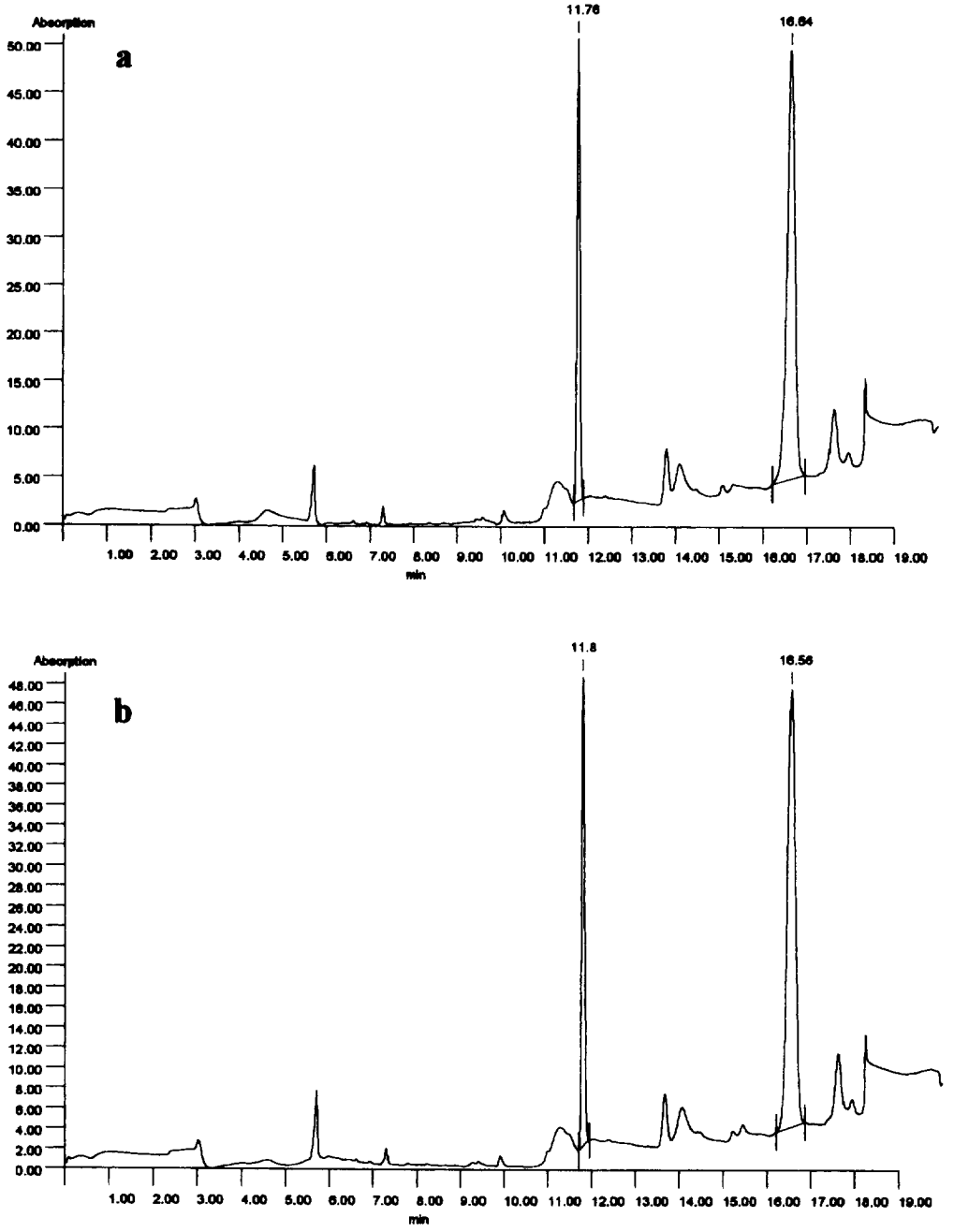

Fig. 2. Human plasma spiked with acetaminophen (t_M = 11.8 min) and salicylate (t_M = 16.6 min), 0.1 mg/ml each; 9th (a) and 12th run (b) of a series of 20 runs; injection time 3.0 s; other conditions as given in Fig. 1. Between runs the capillary was rinsed with a mixture of 50% (v/v) acetonitrile and 50% (v/v) SDS-buffer for 15 min followed by 20 min with running buffer. A stable current and a reproducible separation pattern was obtained.

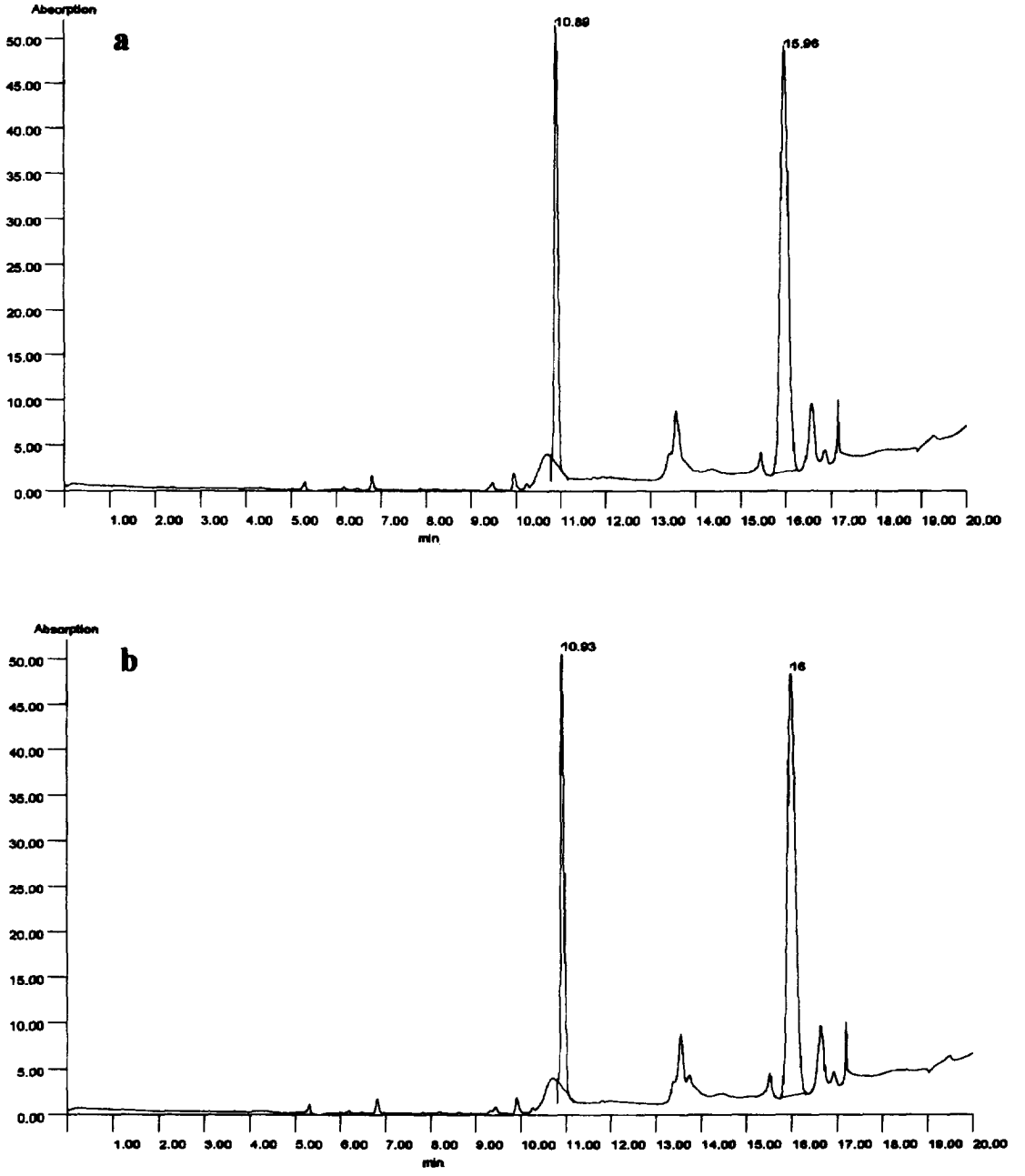

Fig. 3. Human plasma spiked with acetaminophen (t_M=11 min) and salicylate (t_M=16 min); two subsequent runs (a and b) of a series of 85; other conditions as given in Fig. 2. The salicylate peak is free of interferences. However, acetaminophen is overlapping with a minor plasma component which is not observed in Fig. 2. Thereby the reproducibility of the acetaminophen peak area data is disimproved.

only investigate the effectiveness of rinsing procedures. However, most drug concentrations were chosen as relevant for therapeutic drug monitoring. The acetaminophen plasma concentration (100 mg/l) is slightly elevated compared to therapeutic levels (up to 20 mg/l). However, concentrations of 300

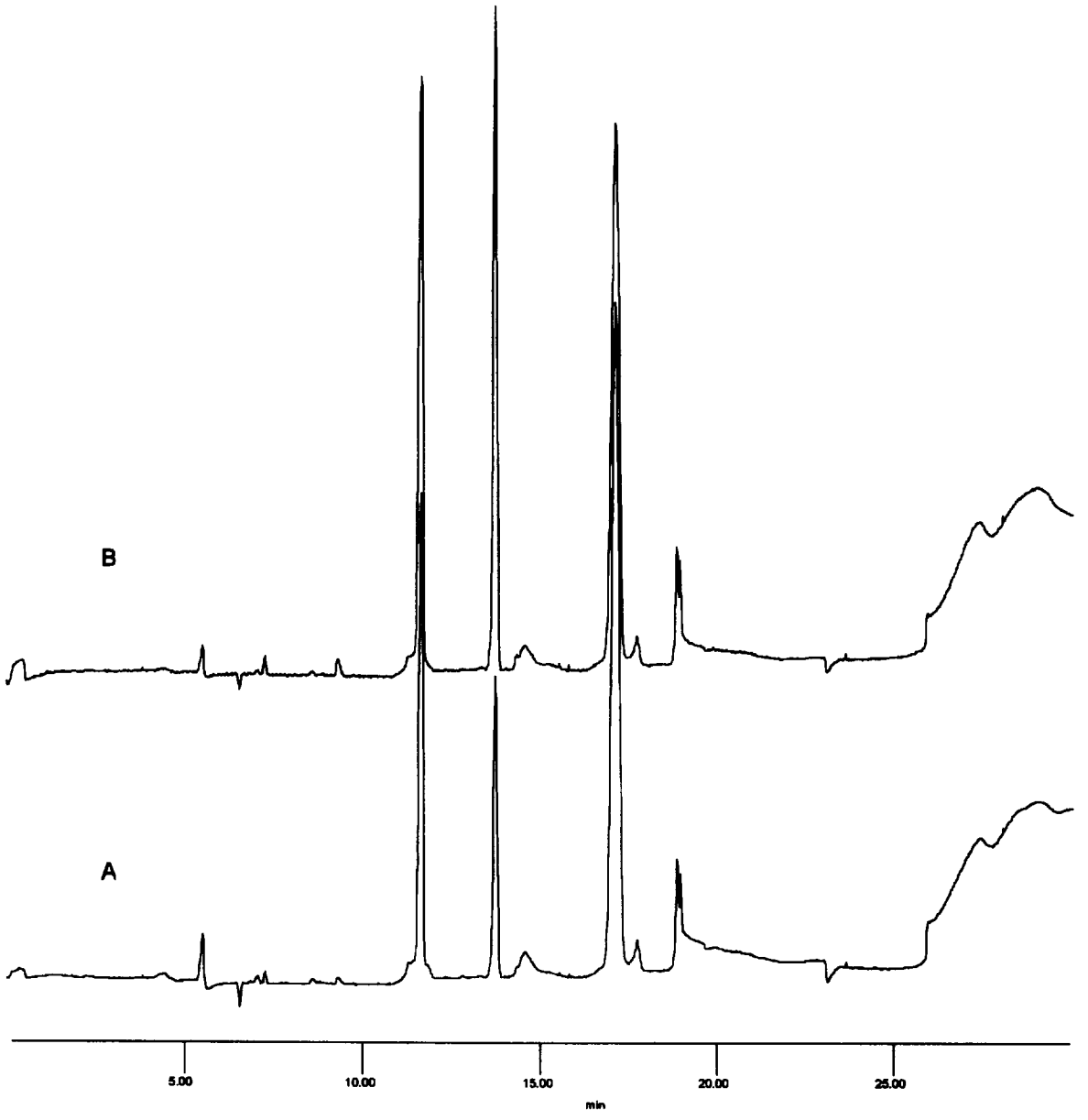

Fig. 4. Human plasma spiked with sulfamethoxazole (first, t_M=11.6 min), tolbutamide (second, t_M=13.7 min) and salicylate (third peak, t_M=17.1 min); injection time 4.0 s; 400 μl of plasma were spiked with 600 μl of drug solutions to give a final concentration of 0.2 mg/ml for each compound; detection was performed at 220 nm (trace A) and at 200 nm (trace B); other conditions as given in Fig. 2.

mg/l have been observed in cases of intoxication. The salicylic acid concentration (86.3 mg/l) corresponds to therapeutic levels (up to 300 mg/l). Sulfamethoxazole was used in a concentration close to the maximum therapeutic level, tolbutamide was doubly concentrated compared to therapeutic levels [13].

In the case of acetaminophen some preliminary investigations at concentrations of 0.01 g/l have been carried out. A reproducibility of 4.46% R.S.D.

for relative peak areas ($n=20$) was obtained (see Table 2). As expected, this value is worse compared to higher concentrations, mostly due to a more unfavorable signal/noise ratio.

4. Conclusions

A reliable method for the determination of acetaminophen and salicylic acid in plasma without sample pretreatment has been established. Day-to-day precisions of about 2.2% for relative peak areas have been obtained ($n>80$). Even for the relatively low concentrations at therapeutic levels a satisfying reproducibility can be obtained. No complete loss of runs and no capillary blockings were observed. This method was successfully transferred from a TSP to a ATI Unicam CE system. The ability of quantifying other drugs because of their migrating within the "analytical window" was shown for other relevant drug substances, i.e., sulfamethoxazole, tolbutamide and trimethoprim.

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