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AUTOMATED ZONE-ELECTROPHORETIC SAMPLE TREATMENT FOR THE ANALYSIS OF BIOLOGICAL SAMPLES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The automation of zone-electrophoretic sample treatment for liquid chromatography is described. The procedure is completely controlled from a liquid chromatograph. The carry-over of proteins from human serum under different experimental conditions was studied. The influence of the presence of proteins in the sample is illustrated with the anionic compound salicylic acid and the increase in selectivity for cationic compounds is demonstrated with the determination of ephedrine, norephedrine and amphetamine in urine.

INTRODUCTION

Recently a new on-line sample treatment method for high-performance liquid chromatography (HPLC) using zone electrophoresis was introduced[1]. This zone-electrophoretic sample treatment (ZEST) method is based on the different migration velocities of compounds in free solution in an applied electric field. The system, consisting of three interconnected pieces of capillary, is coupled on-line to an HPLC apparatus.

The ZEST procedure consists of four stages: (1) all capillaries are filled with electrophoresis buffer; (2) the first (sample) capillary is filled with sample solution; (3) an electric field is applied over the connected capillaries and migration of ionic compounds with the appropriate charge takes place from the sample capillary, through the second (transfer) piece of capillary towards the injection capillary; and (4) after a given time the electrophoresis is stopped. The compounds that have migrated into the

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injection capillary are flushed by the mobile phase towards the column and separation by HPLC can be achieved.

In preliminary studies with a manually controlled system it was shown that it is possible to isolate low-molecular-weight ionic compounds from biological fluids such as blood serum. A theoretical description was given by Kok[2] regarding optimization of the geometry and the electrophoretic conditions.

In this paper the automation and further evaluation of the method and applications to the analysis of serum and urine samples are described. Automation of the method is necessary, as the procedure consist in a sequence of consecutive steps and is sensitive to disturbances caused by manual manipulations. The introduction of an air bubble, for instance, in the capillary system during the injection of the buffer or the sample would interrupt the electrophoretic pathway, and exact timing is important for the reproducibility of the method. The influence of automation on the reliability and reproducibility has been studied.

Two aspects of this method were evaluated: the possibility of achieving the complete isolation of small ionic compounds from proteins in a biological matrix, and the increase in selectivity which is expected from this pretreatment method. For the first aspect, the carry-over of proteins from human serum to the injection capillary under different experimental conditions was studied. The influence of the presence of proteins in the sample was investigated in the determination of the anionic compound salicylic acid.

For the second aspect, the isolation of amphetamine and its derivatives ephedrine and norephedrine as cationic compounds from urine samples was investigated. Because of the relatively high absorption coefficient at low wavelength (about 40 times higher than at 254 nm), these compounds are preferably detected at 210 nm[3]. However, the selectivity of liquid chromatography alone for the determination of these compounds in a complex matrix such as urine is inadequate with detection at 210 nm[4]. Therefore, ZEST was evaluated as a method for increasing selectivity in this application.

EXPERIMENTAL

Chemicals and solutions

Sodium dihydrogenphosphate, disodium hydrogenphosphate, methanol and Folin–Ciocalteu phenol reagent were obtained from Merck (Darmstadt, F.R.G.). Salicylic acid, phthalic acid, copper sulphate, sodium *d*(+)-tartrate, sodium carbonate and ammonium sulphate were purchased from BDH (Poole, U.K.) and benzoic acid from Fluka (Buchs, Switzerland). Norephedrine was obtained from Janssen Chimica (Beerse, Belgium) and *d*-ephedrine from Aldrich (Milwaukee, WI, U.S.A.) The test compound solutions of salicylic acid, amphetamine, norephedrine and ephedrine were prepared daily from concentrated aqueous stock solutions. Pooled human serum was kept at –24°C and was used without any further treatment.

Electrophoresis was carried out in a 0.05 *M* phosphate buffer (pH 6.5) containing 5% methanol. The mobile phase for the determination of salicylic acid was an aqueous solution containing 60% (v/v) methanol and 0.05 *M* phosphate buffer (pH 2). For the determination of amphetamine, ephedrine and norephedrine the mobile phase contained 10% methanol, 0.2 *M* ammonium sulphate and 0.05 *M* phosphate buffer (pH 2).

Apparatus

The chromatographic equipment consisted of a Hewlett-Packard 1090 liquid chromatograph, an autoinjector with a 250-μl syringe, an autosampler and a Model 1040 diode-array detector. The detection wavelength was 230 nm in the determination of salicylic acid and 210 nm in the determination of amphetamine and its derivatives. The column (150 × 4.6 mm I.D.) was packed with Zorbax C_8 (5 μm). Electrophoresis was performed with an LKB 3371 power supply. The whole system was controlled by a Hewlett-Packard HP-85 computer.

The ZEST procedure itself was carried out in a set of three six-port valves connected by PTFE capillaries as described previously[1]. The PTFE capillaries (1/16 in. O.D., 0.48 mm I.D.) were obtained from Chrompack (Middelburg, The Netherlands). The length of the sample, transfer and injection capillaries were 40, 40 and 70 mm, respectively. Taking into account the internal volumes of the valves used, the volume of the sample loaded was 8 μl and 14 μl of buffer solution were injected onto the column.

Conductivities of solutions were measured with a Wayne Kerr B642 autobalance universal bridge with a conductivity cell (cell constant = 1.35 cm^{-1}, Pt black electrodes, length 1 cm)

Protein determination

Protein concentrations were determined by the detection method described by Lowry *et al.*[5], modified for small amounts. After electrophoresis the injection capillary was flushed with water, 250 μl of effluent were collected in a tube and 2 ml of a solution containing 2% Na_2CO_3, 0.1 *N* NaOH, 0.01% $CuSO_4$ and 0.02% sodium tartrate in water were added. After 10 min 200 μl of Folin–Ciocalteu reagent, diluted 1:1 with water, were added and mixed. After 30 min the absorption was measured at 750 nm. For calibration the same determination was carried out with dilute serum solutions pipetted directly into the reaction tubes.

For detection a Philips PU 8700 spectrophotometer was used.

RESULTS AND DISCUSSION

Automation and development

The automated set-up is shown in Fig. 1. The air-pressure-driven switching valves and the power supply, the injection syringe and the autosampler are controlled

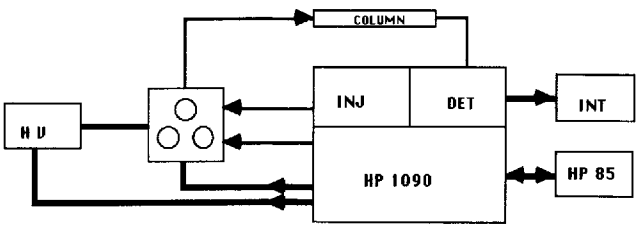

Fig. 1. Schematic diagram of set-up for automated zone electrophoretic sample treatment. INJ = injector; HV = high-voltage power supply; INT = integrator; DET = detector.

TABLE I

TIME SCHEDULE FOR ZONE ELECTROPHORETIC SAMPLE TREATMENT

Electrophoresis time, 16 min.

Time (min)	*Event*	*Valve*	*HV*
0.00	Program loading		
0.50	Flushing with buffer	PREP	
3.00	Sample injection	LOAD	
5.50	Start of electrophoresis	PHOR	On
6.00	Flushing with buffer		
21.50	End of electrophoresis		Off
21.55	Injection on column	ELUT	
22.00	End		

from the chromatograph. A typical timing schedule, a simplified representation of the program retrieved from disk, is shown in Table I.

The procedure starts by flushing of the capillaries with 2 × 200 μl of buffer solution. It appeared to be necessary to use this large volume in order to avoid carry-over of previous samples. For safety reasons (high voltage) the electrophoretic system is contained in a separate compartment, so that the length of the tubing between the injection syringe and the switching valves has to be considerable. After changing the valve positions, the sample loop is filled by injecting 2 × 200 μl of the sample solution. A decrease in the amount of sample resulted in irreproducible results. While electrophoresis is in progress, after another change in the positions of the valves, the connecting tube is flushed with buffer solution to wash out remaining sample solution. When the electrophoresis is terminated, the valves are switched to the injection position and compounds that have migrated to the injection capillary are separated on the column. During the separation the next ZEST procedure starts.

The electrical field strength in the capillaries was determined by measuring voltage current relationships with different valve positions. With the maximum voltage of the power supply (1350 V) the electrical field strength is about 50 V cm^{-1} in the 0.48 mm I.D. capillaries. A large part of the voltage is lost in the tubing connecting the electrode vessels to the valve system. To examine the correctness of the connections of the capillaries to the valves, the measured currents were compared with calculated values. With 0.05 *M* phosphate buffer (pH 6.5) containing 5% methanol the current measured immediately after turning on the high-voltage supply at 1350 V was 0.37 mA. Calculation of the current, using a measured conductivity value of 5.1 ohm^{-1} cm^{-1} for this buffer, yields 0.36 mA. The fair agreement between the current outputs shows that there are no major disturbances to the electrophoretic pathway.

During the electrophoresis the current increased, until after about 10 min a steady-state level of 0.45 mA was reached using the buffer described above. The current increase is caused by the increase in the temperature in the capillaries, which can be calculated from these figures to be in the range 10–15°C.

The reproducibility of the automated system was evaluated using standard solutions of organic acids as model compounds. In Fig. 2 the results of recovery studies are shown. The recoveries were calculated by comparing peak areas obtained

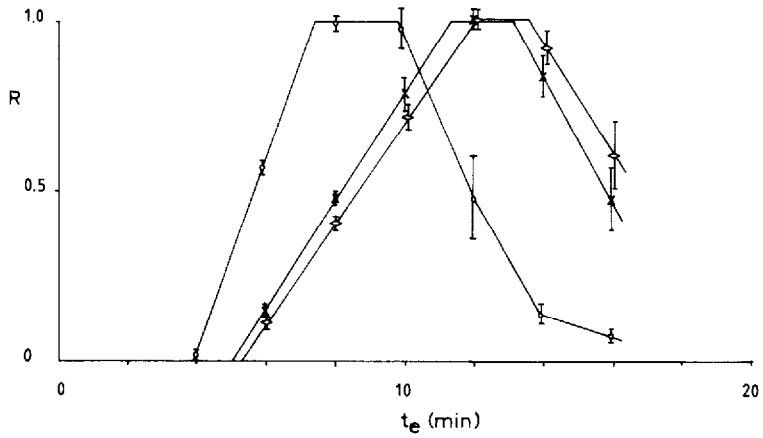

Fig. 2. Recovery (R) of (○) phthalic, (◇) benzoic and (×) salicylic acid from 0.05 M phosphate buffer (pH 6.5) *versus* time of electrophoresis (t_e). Each point represents the mean and standard deviation of three experiments. Mobile phase, 0.05 M phosphate buffer (pH 2)–methanol (60:40).

after ZEST with those after direct injection of the standard solutions. Fig. 2 shows that phthalic acid, which is doubly charged in the buffer used at pH 6.5, migrates faster than the monocarboxylic benzoic and salicylic acids. Partial separation can be achieved. As the injection capillary is longer than the sample capillary, a range of electrophoresis times is available for complete recovery of a compound. The reproducibility is considerably better on the plateau than on the slopes of the recovery curves. The relative standard deviation was 2% on this plateau, whereas it could be more than 20% on the slopes. Apparently, reproducibility of the migration speed is an important factor.

Protein migration

Experiments were carried out to investigate the carry-over of the proteins from the serum samples. The electrophoresis was performed in 0.05 M phosphate buffer and the serum sample was diluted with buffer and water (serum:buffer:water = 1:8:1) to avoid conductivity disturbances at the sample zone boundaries. The amount of proteins which reached the injection loop after electrophoresis was determined at different pH values and different directions of the electrical field. After electrophoresis the loop was purged with 250 μl of an aqueous solution and collected in a tube directly after the injection loop and reagents were pipetted into the tubes. Recoveries in the ZEST procedure were obtained by comparing units of absorption. Detection of less then 1 μg/ml of protein was possible.

Fig. 3 shows the results of the protein electrophoresis experiments. At pH 2 proteins began to enter the injection loop after electrophoresis for about 8 min when the electrophoresis was carried out in the direction of the negatively charged electrode. At pH 2 proteins are highly positively charged and migrate rapidly to the injection loop. Therefore, at this pH the isolation of small cations will be difficult. After 20 min virtually all proteins have migrated to the loop. Complete carry-over corresponds to 67 μg of serum proteins injected (8 μl of diluted serum).

Comparable results are obtained with a buffer of pH 11 with electrophoresis in

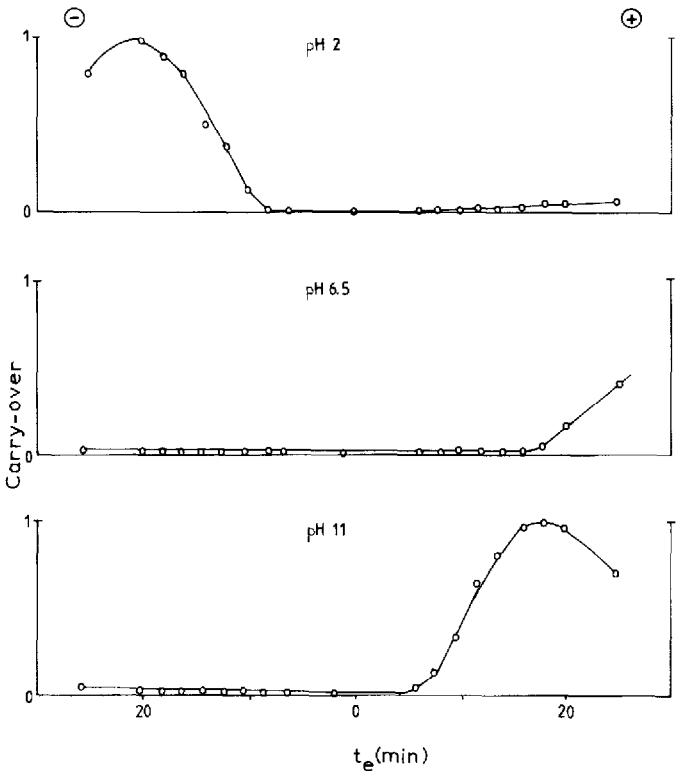

Fig. 3. Carry-over of blood serum proteins as a function of the electrophoresis time in the direction of the positive electrode (right-hand side) and the negative electrode (left-hand side), at different pH values of the buffer.

the direction of the positive electrode. Carry-over of proteins started after 5 min of electrophoresis, so that the isolation of small anions will be difficult.

At pH 6.5 electrophoresis can be carried out in both electrical field directions up to 18 min before the first proteins enter the injection loop.

Salicylic acid in human blood serum

As a result of the studies mentioned above and preliminary studies[1] phosphate buffer of pH 6.5 was chosen for ZEST of the anionic drug salicylic acid. Fig. 4 shows the results of recovery studies of salicylic acid from standard buffer solutions and from human blood serum. The recovery from serum samples is not complete, which indicates that salicylic acid undergoes a protein binding process. The maximum recovery from serum is 85% compared with standard solutions. However, it is not yet clear how this relates to the extent of protein binding of the drug.

The calibration graphs for salicylic acid in the range 10^{-5}–$5 \cdot 10^{-4}$ *M* (Fig. 5), the concentration range in serum found after a therapeutic dose, are linear (buffer and serum, $r = 0.999$). The reproducibility was better than 3% for standard solutions ($n = 7$) and 6% for serum samples ($n = 4$).

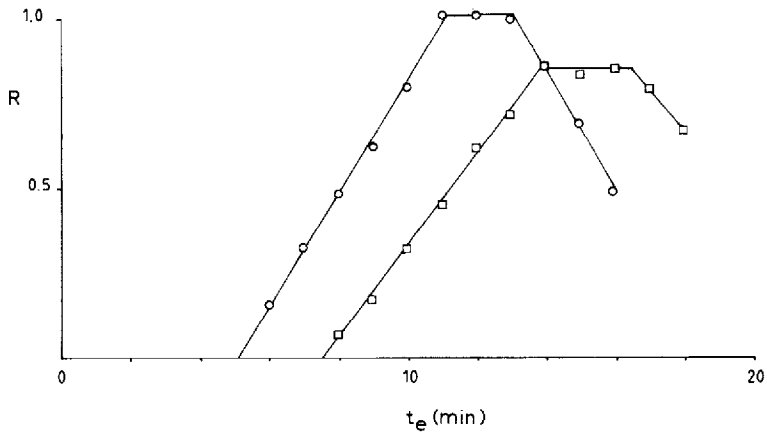

Fig. 4. Recovery of salicylic acid (10^{-4} *M*) from (○) 0.05 *M* phosphate buffer (pH 6.5) and (□) human blood serum *versus* electrophoresis time. Each point represents the mean of three experiments.

Amphetamines in urine

The applicability of ZEST to the prepurification of urine samples with cationic analytes is demonstrated with amphetamine and two of its major metabolites, ephedrine and norephedrine. The pK_a values of these compounds are 9.8, 9.7 and 9.4, respectively[6].

Urine samples were diluted 1:1 with water to avoid high conductivity disturbances at the sample zone boundaries. Electrophoresis was performed in phosphate buffer of pH 6.5, resulting in migration of the positively charged molecules to the

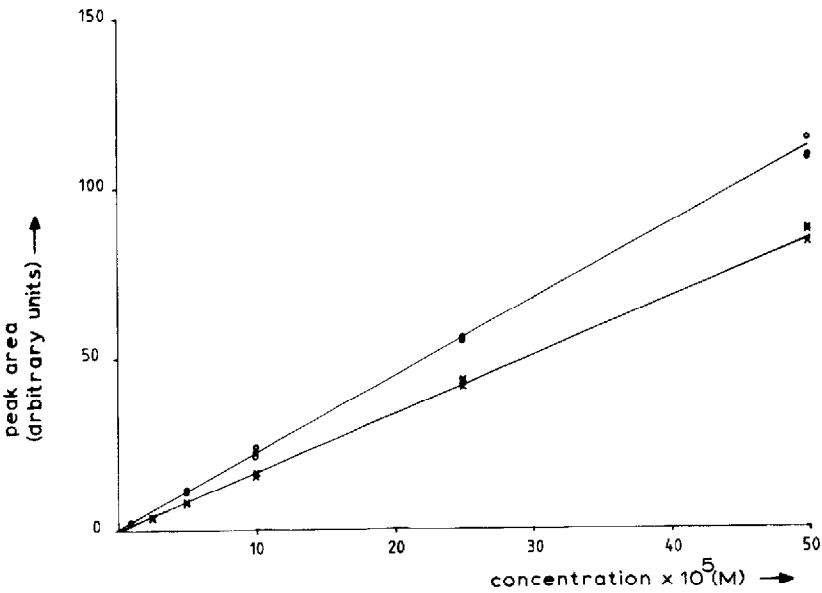

Fig. 5. Calibration graphs for salicylic acid in (○) 0.05 *M* phosphate buffer (pH 6.5) and (×) blood serum.

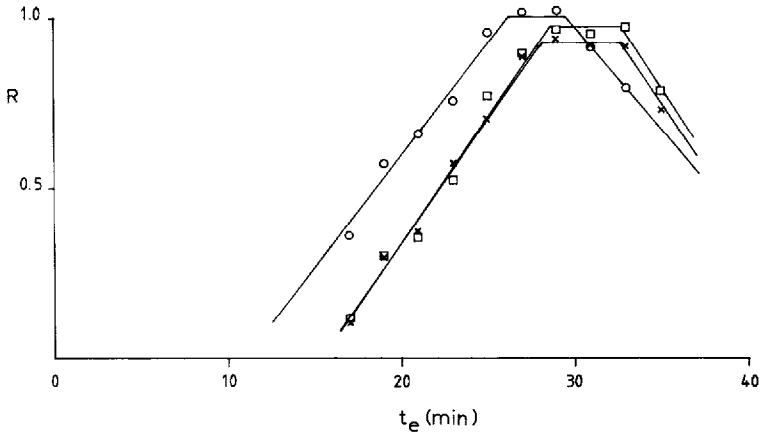

Fig. 6. Recovery of (○) amphetamine, (□) norephedrine and (×) ephedrine *versus* time of electrophoresis; standard solutions $5 \cdot 10^{-5}$ *M* in 0.05 *M* phosphate buffer (pH 6.5). Each point represents the mean of three experiments.

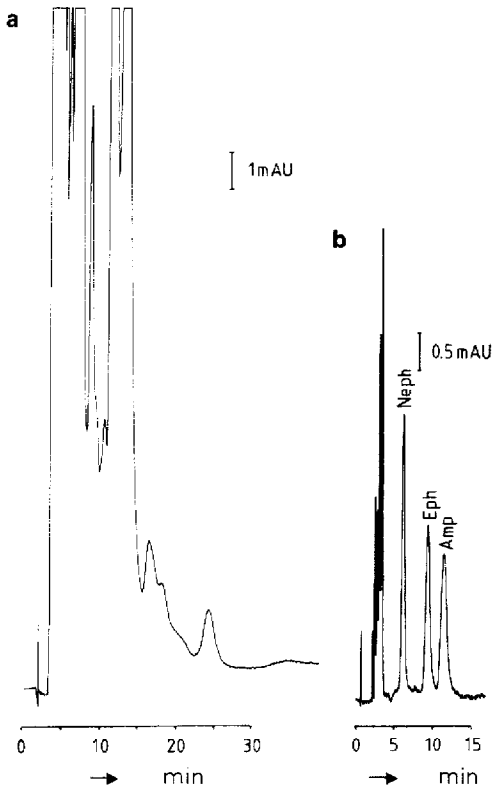

Fig. 7. Chromatogram of a urine sample spiked with amphetamine (Amp), norephedrine (Neph) and ephedrine (Eph) ($5 \cdot 10^{-5}$ *M*). (A) Untreated sample; (B) after 29 min of electrophoresis (1350 V). For experimental conditions, see text.

negative electrode. Dilute urine samples were spiked with amphetamine, ephedrine and norephedrine, all at $5 \cdot 10^{-5}$ *M*.

Fig. 6 presents the recovery curve of amphetamines from buffer solutions. The optimum electrophoresis time is 29 min under these conditions for the three compounds. In comparison with untreated urine samples the selectivity of the determination is considerably improved (Fig. 7). No interfering substances were found in urine although a non-selective wavelength of 210 nm is used.

CONCLUSIONS

ZEST can be a powerful pretreatment method for isolating anionic and cationic analytes from biological samples prior to liquid chromatography. The ZEST procedure can be automated and is therefore suitable for routine analysis.

For the analysis of serum or plasma samples an important feature of the ZEST method is that proteins can be removed quantitatively on-line. Only for the isolation of very weak acids ($pK_a \gg 7$) or very weak bases ($pK_a \ll 7$) for which an electrophoresis buffer with a pH strongly different from physiological pH should be used can carry-over of proteins not be avoided. In comparison with other techniques that allow direct injection of serum or plasma, such as precolumn techniques[7,8] or chromatography with internal-surface stationary phases[9], ZEST offers an increase in the selectivity for ionic compounds. This selectivity is also maintained in the analysis of urine samples.

It has been shown[2] that the sample capacity and the time necessary for electrophoresis in ZEST are interrelated. Preconcentration of compounds from large volumes of samples will generally not be possible. However, owing to the selectivity of the pretreatment, detection can often be performed at low wavelength, with a high sensitivity. However, instrumental improvements are necessary to decrease the time of electrophoresis and/or increase the sample capacity. Investigations in this direction are now being carried out.

Measurements of the recovery of a compound from a biological sample could give an indication of the extent of protein binding. However, for the establishment of quantitative relationships between recovery and protein binding further investigations are necessary.

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