

# DIRECT DETERMINATION OF VALPROATE IN SERUM BY ZONE ELECTROPHORESIS - ISOTACHOPHORESIS ON A COLUMN-COUPLING CHIP

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## INTRODUCTION

Zone electrophoresis (ZE) on-line coupled with isotachopheresis (ITP) sample pretreatment (ITP-ZE) is a CE technique very convenient to trace analysis applications. This combination, integrating the sample pretreatment with the separation, is in a straightforward way transferable to a chip format. Its analytical advantages in the separations on the chips were already demonstrated.

This feasibility study was aimed at investigating potentialities of ITP-ZE on a poly(methylmethacrylate) chip with the coupled separation channels (CC) and in-column conductivity detection to a direct determination of drugs in serum. Valproic acid (an antiepileptic drug), having a therapeutic range of 50-100 mg/l (0.35-0.69 mmol/l) and a toxic level of 130 mg/l (0.90 mmol/l), was a test analyte in our study while reference serum samples served as proteinous matrices.

## ITP-ZE SEPARATIONS ON THE CHIP

A composition of the electrolyte system in which the ITP-ZE separations of model and serum samples were carried out is given in Table 1. Here, the ITP electrolyte conditions were chosen in such a way that valproate migrated in the ITP stack while the proteinous serum macroconstituents migrated zone electrophoretically in the terminating zone (to prevent the concentration and, potentially, a significant precipitation of the proteins on the chip).

The working conditions in the ITP stage of the combination were chosen in such a way that ITP performed a multitask sample pretreatment:

- 1) the separation of the analyte from the serum matrix and its concentration into a narrow ITP band,
- 2) a removal of the matrix constituents migrating in the ITP stack from the separation compartment of the chip,
- 3) an ITP stacking of the drug released on a continuous electrophoretic decomposition of the drug-protein complex.

A high sample load capacity, closely linked with the use of ITP in the first separation stage, made possible to inject the samples with the aid of a 0.95  $\mu$ l sample channel. Concomitantly, the working conditions in the ZE stage favored the resolution of valproate from the stacked matrix constituents and, at the same time, its sensitive detection by the conductivity detector.

Table 1 Electrolyte system

ITP	water	Solvent	water	ZE
Solvent	water	Solvent	water	
Leading anion	Cl <sup>-</sup>	Carrier anion	MES	
Concentration (mmol/l)	10	Concentration (mmol/l)	10	
Counterion	HIS	Counterion	HIS	
EOF suppressor	mHEC	EOF suppressor	mHEC	
Concentration (% w/v)	0.05	Concentration (% w/v)	0.1	
pH	6.1	pH	5.5	
Terminating anion	MES			
Concentration (mmol/l)	5			
Counterion	HIS			
EOF suppressor	mHEC			
Concentration (% w/v)	0.05			
pH	6.0			

HIS = histidine, mHEC = methylhydroxyethylcellulose, MES = 2-(N-morpholino)etanesulfonic acid

## QUANTITATION OF VALPROIC ACID IN SERUM

Using a 0.95  $\mu$ l sample volume we reached a 0.2-0.4  $\mu$ mol/l concentration limit of detection for valproate from the response of the conductivity detector in the ZE stage of the combination (slight differences for the model serum matrix and serum were found).

A complete ITP resolution of valproate from the serum constituents was obtained when a 70 times diluted serum was loaded into the injection channel of the chip (this corresponded to the load of a 13.5 nL of the undiluted serum).

Repeatabilities of the ITP pretreatment times, the total migration times (the time included the ITP sample pretreatment and ZE separation) and peak areas of valproate are summarized in Table 2.

The quantitation of valproate in serum was possible when its concentration in the loaded sample was 2  $\mu$ mol/l (the lowest concentration for which the calibration data were obtained [Table 3]). This value is lower than the lowest value of the therapeutic range of valproate in serum (5  $\mu$ mol/l when a 70-fold dilution of serum is assumed). Electropherograms in Fig. 3 illustrate the capabilities of the ITP-ZE on the chip in such a situation.

Table 3 Parameters of the regression equations ( $y = ax + b$ ) for the calibration graphs of valproate

Parameter	a	b	r	Serum	Dilution of serum	n
(mV.l/mol.s)	(mV.s)					
	$3.398 \cdot 10^7$	-21.5	0.9997	M	100	16
	$3.321 \cdot 10^7$	-19.3	0.9991	M	50	16
	$3.521 \cdot 10^7$	-40.0	0.9990	M	20	20
	$3.783 \cdot 10^7$	-47.8	0.9961	P	70	12
	$3.391 \cdot 10^7$	-47.8	0.9911	P	70	15

a = intercept, b = slope of the calibration line, r = correlation coefficient, x = concentration of the analyte, y = peak area, M = model sample serum, P = pathological serum, n = number of data points. The calibration data were measured for  $2 \cdot 10^{-5}$  -  $5 \cdot 10^{-5}$  mol/l concentration of valproate.

## PRE-COLUMN SAMPLE HANDLING

The filtration of the diluted serum sample through a membrane filter (a 0.45  $\mu$ m pore size) was found to be the only sample handling operation needed before its loading on the chip. From the practical point of view it is important that no disturbances attributable to the precipitation of proteins from the loaded samples on the chip were detected.

A total recovery of valproate from serum samples in its direct ITP-ZE determination was 92% (Fig. 4) and a repeatability of this value characterized 2.9-3.7% RSD values. It was somewhat surprising that when the sample filtration was omitted, the recovery value dropped (D, in Fig. 4) to about 50%. In this context we should also note that in comparative experiments, using a currently preferred acetonitrile deproteinization, we obtained the recovery values below 50% (C1 and C2, in Fig. 4).

## INSTRUMENTATION

The separations on the CC chips were performed in a laboratory constructed CE equipment (see a scheme in Fig. 1).

An electrolyte and sample management unit (E&SMU, in Fig. 1), connected via 300  $\mu$ m I.D. FEP capillary tubes to the inlets of the channels on the chip (see Fig. 2), consisted of the following operational elements:

Valves (V1, V2, V6 and V5, in Fig. 1), opening the inlets on the chip on filling the channels with the electrolyte and sample solutions (they were closed during the separations).

Micropumps (P1, P2, P5, P6, in Fig. 1), connected to the inlets of the corresponding valves, for deliveries of the electrolyte and sample solutions to the channels before the CE run.

A permanently opened outlet channel of the chip, connected to a waste container (W, in Fig. 1).

An electronic and control unit (E&CU, in Fig. 1) provided the following functions:

- Delivered the driving current,
- Measured the conductivity with the aid of platinum detection sensors, sputtered on the cover of the channels of the chip,
- Controlled the ITP-ZE runs,
- Interfaced the CE equipment to a PC computer.

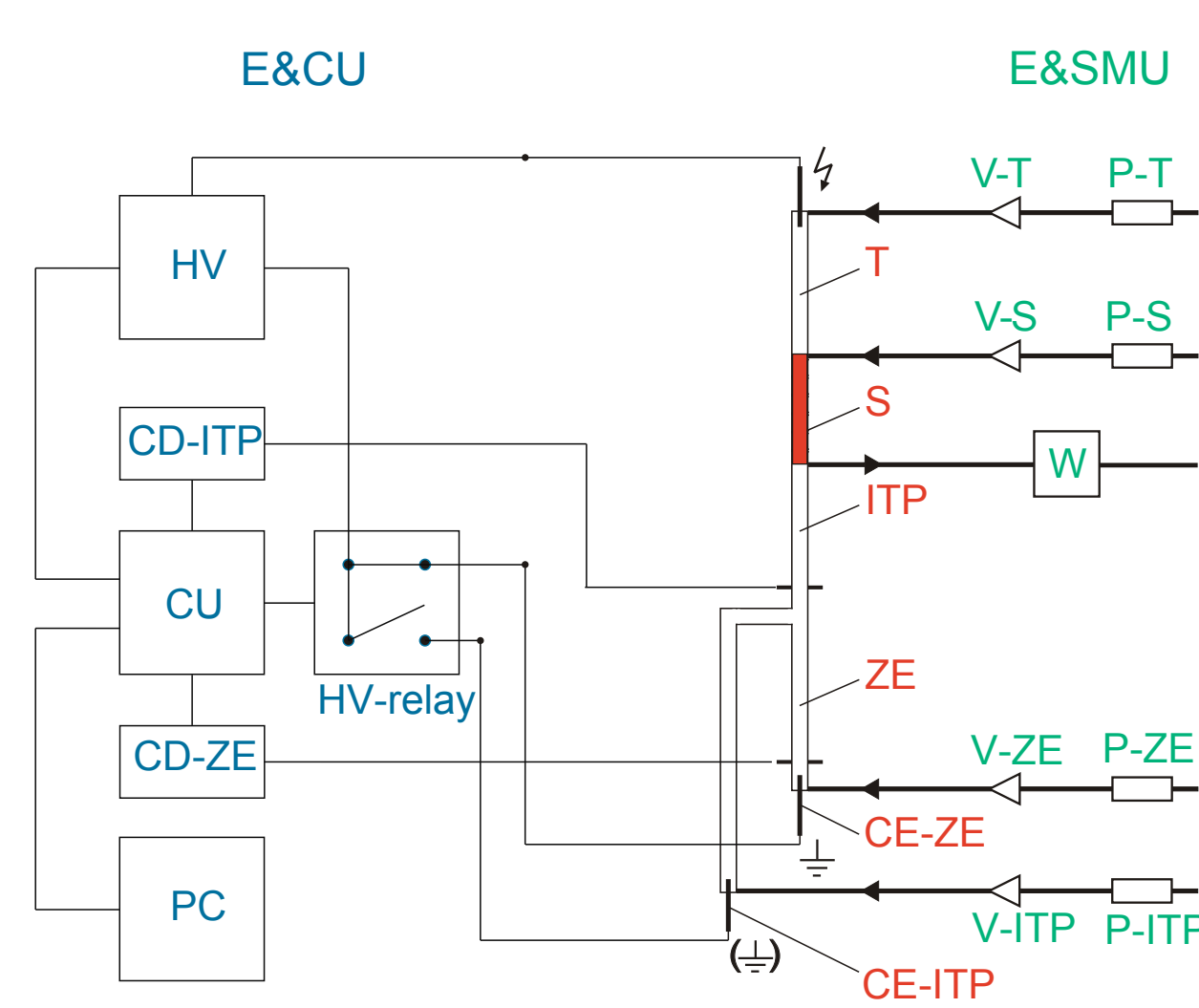


Figure 1 A scheme of the CE equipment provided with the CC chip

Electronic and control unit (E&CU): CU = control unit; HV = high-voltage power supply (0-50 A, 0-5 kV); CD-ITP, CD-ZE = conductivity detectors for the ITP and ZE separation channels, respectively; HV-relay = a high-voltage relay switching the direction of the driving current in the separation compartment; CE-ITP, CE-ZE = counter-electrodes for the ITP and ZE separation channels, respectively.

Electrolyte and sample management unit (E&SMU): V-ITP, V-ZE, V-S, V-T = valves for the inlets to the ITP, ZE, sample and terminating channels of the chip, respectively; W = waste container; P-ITP, P-ZE, P-S, P-T = micropumps for filling the ITP, ZE, sample and terminating channels with the electrolyte and sample solutions, respectively.

Table 2 Repeatabilities of some parameters of valproate in serum

valproate (mol/l)	$t_{IP}$ (s)	$t_{IP+ZE}$ (s)	A (mV.s)	Serum	n
$1 \cdot 10^{-5}$	Average	242.6	560.1	306.1	50 times
	SD	3.2	3.1	5.3	diluted serum M
	RSD (%)	1.3	0.5	1.7	
$1 \cdot 10^{-5}$	Average	241.2	557.1	307.7	50 times
	SD	4.2	6.6	12.4	diluted serum M
	RSD (%)	1.7	1.2	4.0	
$7 \cdot 10^{-6}$	Average	233.91	556.1	184.7	100 times
	SD	4.85	9.8	6.2	diluted serum N, P
	RSD (%)	2.1	1.8	3.3	

$t_{IP}$  = time of the entering of preset zone to the CD1 in ITP stage,  $t_{IP+ZE}$  = total migration time of valproate, A = peak area of valproate, n = number of parallel measurements, M = model sample serum, P = pathological serum, N = normal serum

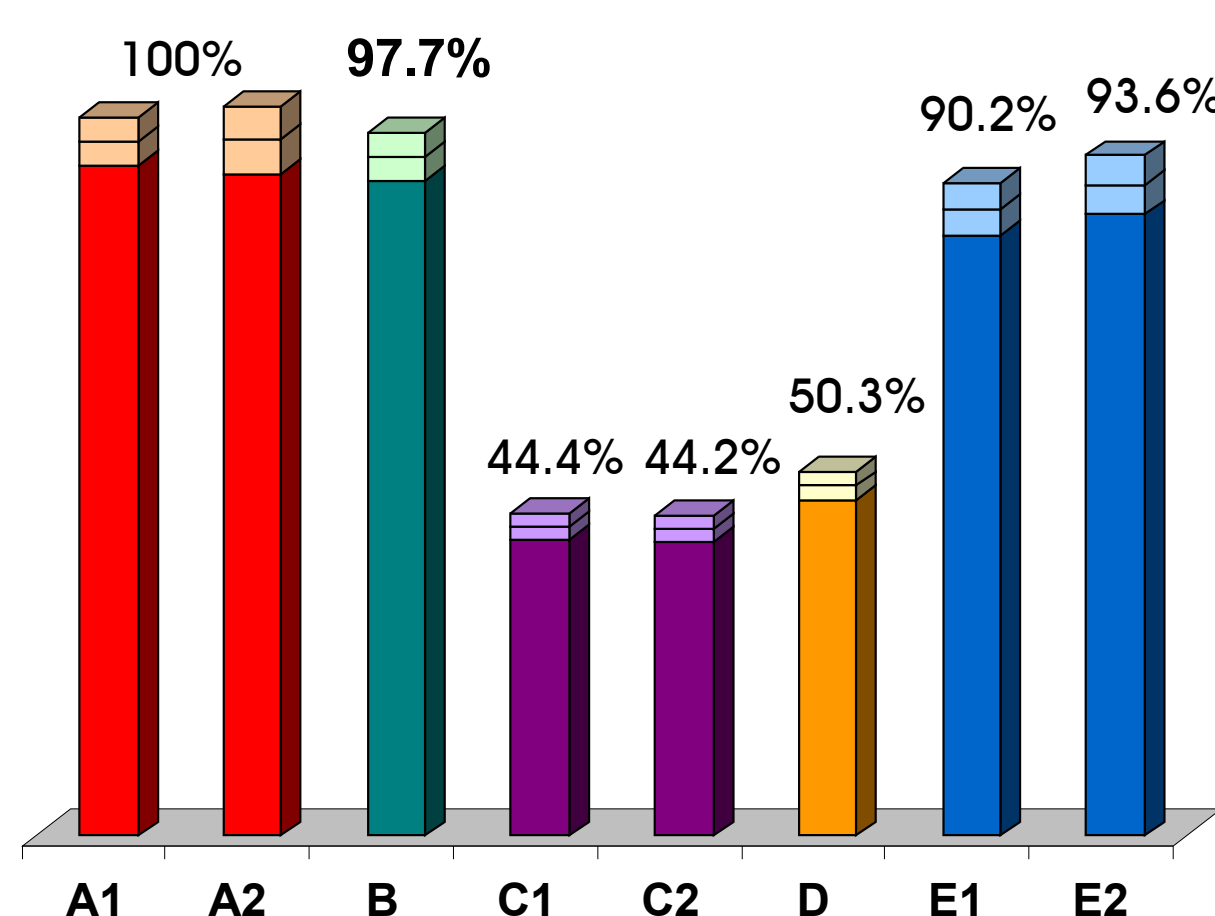


Figure 4 Recoveries of valproate (present in the loaded samples at a 50  $\mu$ mol/l concentration) for various pre-column sample preparation procedures

A1, A2 = a model matrix sample (without proteinous constituents) spiked with valproate at a 50  $\mu$ mol/l concentration (this sample was a reference in the calculations of the recovery values); B = a pathological serum sample spiked with valproate at a 50  $\mu$ mol/l concentration (valproate was added to the sample after its deproteinized by acetonitrile); C1, C2 = as B, only valproate was added to the sample before its deproteinization; D = a pathological serum sample spiked with valproate at a 50  $\mu$ mol/l concentration (the sample was loaded directly after a 100-fold dilution); E1, E2 = as D, only the diluted serum sample was filtered by a filter of a 0.45  $\mu$ m pore size before the loading onto the chip.

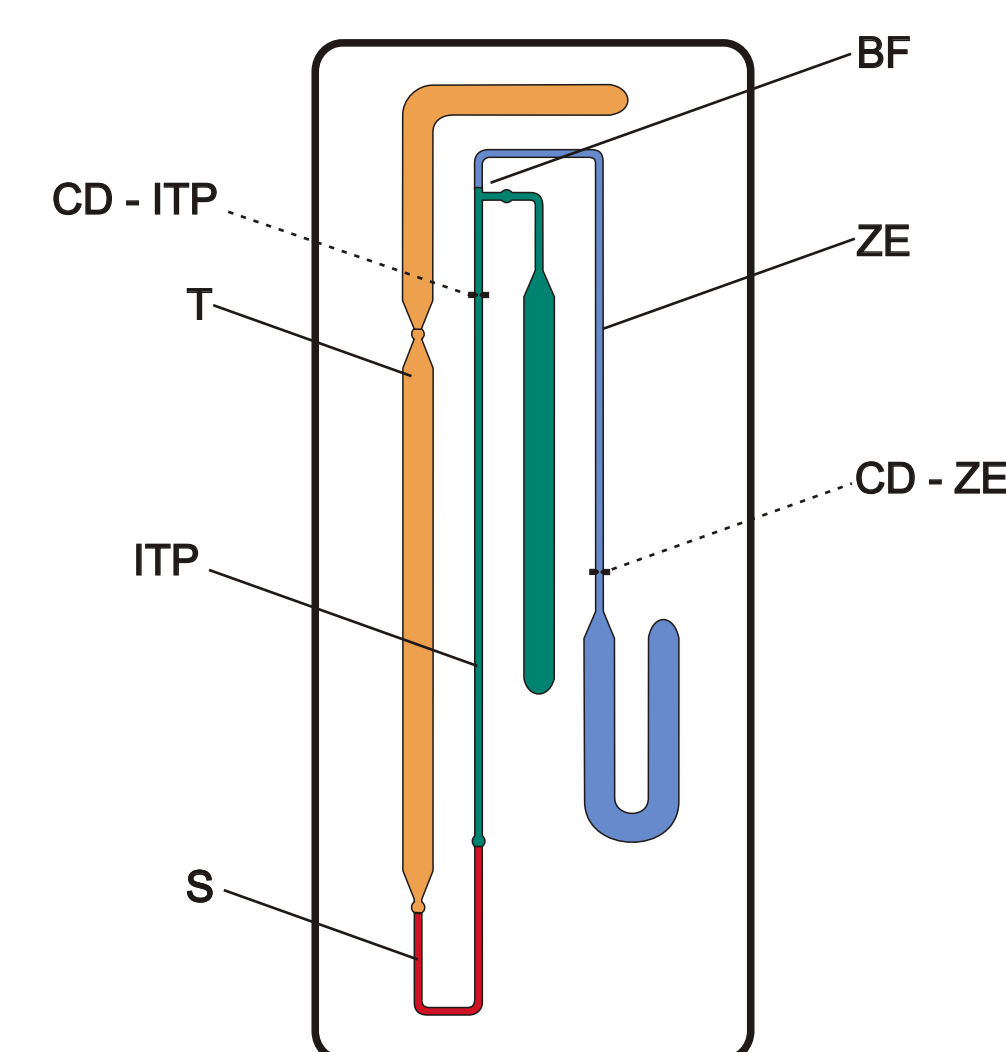


Figure 2 CC chip

T = terminating electrolyte channel; S = sample injection channel (a 0.95  $\mu$ l volume; 24 x 0.2 x 0.2 mm [length, width, depth]); ITP = ITP separation channel (a 3.04  $\mu$ l volume; 76 x 0.2 x 0.2 mm [length, width, depth]) with a platinum conductivity sensor (connected to CD-ITP); ZE = ZE separation channel (a 1.68  $\mu$ l volume; 42 x 0.2 x 0.2 mm [length, width, depth]) with a platinum conductivity sensor (connected to CD-ZE); before = bifurcation plane.

## SAMPLES

A model matrix sample, representing a low-molecular ionic matrix of serum, consisting of sodium salts of chloride ( $8.4 \cdot 10^{-2}$  mol/l), lactate ( $1 \cdot 10^{-3}$  mol/l) and phosphate ( $1 \cdot 10^{-3}$  mol/l).

Normal and pathological reference serum samples.

When needed, the serum sample was deproteinized by acetonitrile (the sample mixed with the solvent in a 1:1 volume ratio) with a removal of the proteinous precipitate by centrifugation (10000 rpm for 20 minutes).

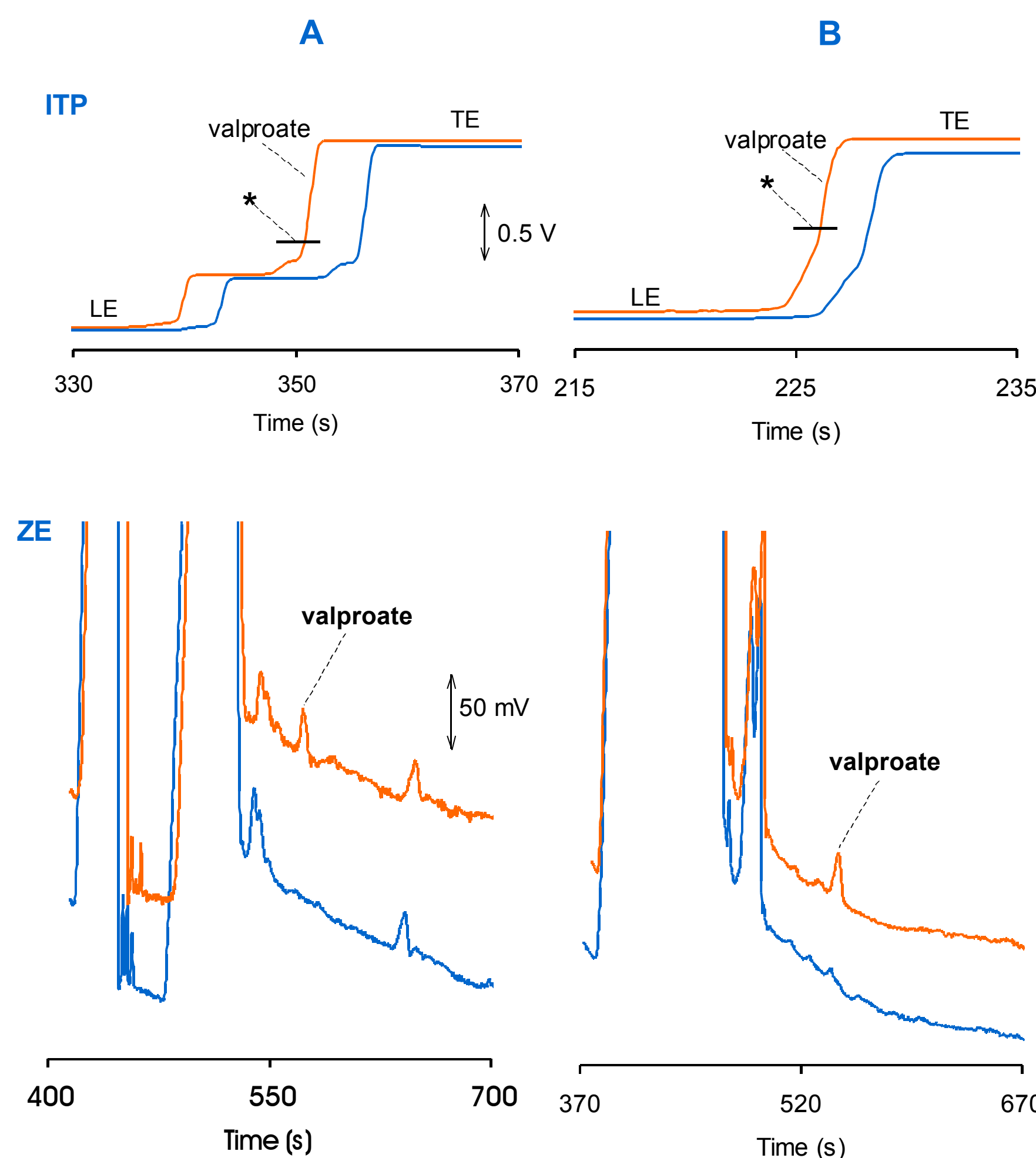


Figure 3 Electropherograms from ITP-ZE separations of model (a) and serum (b) samples spiked with valproic acid at 50  $\mu$ mol/l concentrations

The separations were carried out in the electrolyte system described in Table 1 with 7.5 and 5  $\mu$ A driving currents in the ITP and ZE stages, respectively. The samples were diluted 70-fold before the analysis. The serum sample was, in addition, filtered by a disposable membrane filter (a 0.45  $\mu$ m pore size).

Asterisks on the isotachopherograms mark the position from which the constituents present in the ITP stack were transferred to the ZE stage. Electropherograms from the ZE stage as obtained for unspiked samples serve as references to which the spiked samples were related.

## CONCLUSIONS

- The ITP-ZE combination on the CC chip offers a simple and rapid (less than 10 minutes) procedure to a direct determination of valproate in serum. The serum dilution and filtration were the only required pre-column sample handling operations.
- The conductivity detection in the ZE stage of the combination provided a 0.2-0.4  $\mu$ mol/l concentration limit of detection for valproate. Such a detection sensitivity covered the therapeutic range of valproate in serum despite the fact that the serum samples had to be 50-70 fold diluted before the analysis.
- It seems reasonable to assume that ITP-ZE on the CC chip can be extended to the direct determination of other drugs in serum. However, enhanced detection sensitivities and/or increased sample loadabilities of the ITP stage may become essential in reaching the required concentration levels for some drugs.