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**Citation for published version (APA):**

Claessens, H. A., Lemmens, A. A. G., Sparidans, R. W., & Everaerts, F. M. (1988). Pretreatment of body fluids by preparative isotachophoresis prior to chromatographic analysis. *Chromatographia*, 26(1), 351-358.  
<https://doi.org/10.1007/BF02268180>

**DOI:**

[10.1007/BF02268180](https://doi.org/10.1007/BF02268180)

**Document status and date:**

Published: 01/01/1988

**Document Version:**

Publisher's PDF, also known as Version of Record (includes final page, issue and volume numbers)

**Please check the document version of this publication:**

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
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# Pretreatment of Body Fluids by Preparative Isotachopheresis Prior to Chromatographic Analysis

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## Key Words

Column liquid chromatography  
Isotachopheresis  
Sample pretreatment

## Summary

This study focusses attention on the possibilities of preparative isotachopheresis (ITP) as a sample pretreatment technique prior to liquid chromatographic (HPLC) analysis. The increased demand for accurate and less time consuming analysis necessitates that sample pretreatment procedures, should be developed in parallel with other improvements (e.g. in detection and separation) which can be observed. The preparative isotachopheresis was performed on gel slabs and the zones of interest were subsequently cut out, desorbed and the desorbates analyzed by HPLC. In this study satisfactory recoveries of between 85–90% with a standard deviation of 1–5% were observed for blank experiments. For spiked serum and urine samples the recoveries in general decreased with decreasing spiked drug concentrations. These observations are discussed in this paper.

## Introduction

In many application areas of High Performance Liquid Chromatography (HPLC), i.e. environmental, food and biochemistry, the demand for accurate, less time consuming analytical procedures at the nanogram or subnanogram level is increasing.

The limited separating power and detection sensitivity of HPLC systems often necessitate a sample pretreatment procedure prior to separation. Analytical procedures consist of a number of sequential steps such as sample pretreatment, separation, detection and data handling. Of these sample pretreatment, an integral part of most analyses, is often the weakest part of the whole procedure.

Since the weakest part of a chain mostly determines the final result, it is important that sample pretreatment is integrated with other developments that can be observed in separation and detection techniques. Usually sample pretreatment procedures consist of isolation of components of interest and in a number of cases also a preconcentration of the components in order to meet the required detection limit.

In general sample pretreatment procedures should meet the following conditions:

- i. high sample capacity.
- ii. the method must be selective for the components under study, and avoiding interfering components.
- iii. the recoveries must be high and reproducible.
- iv. preferably, the procedure should take up little time and be automated.

Many pretreatment procedures for HPLC analysis are applied at present. In HPLC liquid-liquid (LL), liquid-solid (LS) and solid-phase (SP) isolation procedures are widely applied as sample pretreatment methods. LL-isolation methods are in general selective due to the range of options with which the transfer of the components under study from the sample phase to the extractant phase may be manipulated by e.g. type of the solvents, pH, polarity, ionic strength and ion pairing agents [1–5]. LL-isolation procedures are in general laborious, difficult to automate and often ending up in relatively large volumes of extractant, after which a concentration step becomes necessary.

In LS-pretreatment methods, which in general are carried out in HPLC columns, the sample is fractionated in distinct groups of similar components due to specific component to stationary phase interactions [5–10], the concentrations of the components are reduced due to the chromatographic dilution and can be performed either in an on-line or off-line mode [11–16].

In SP-methods many adsorbents, some of them also applied in analytical HPLC, are used to isolate the components of interest and to get rid of interfering components. These methods are often based on non-specific sorbent to components interactions and are often complicated by matrix

effects and limited sample capacity [17–20]. SP-techniques are applied as trace enrichment procedures or to change the solvent in which the components are dissolved [21, 22].

As mentioned earlier, the increasing demands for accurate and sensitive analysis procedures urge the development of improved sample pretreatment procedures to run parallel with the developments in separation and detection areas.

This prompted us to investigate the extent to which electrophoretic techniques may contribute to the selective isolation of ionic components prior to HPLC separation. As is common knowledge, electrophoretic separation techniques are based on the migration of ionic components in an electric field under well controlled experimental conditions. Electrophoretic techniques are principally selective isolation procedures for ionic substances.

The relationship between the effective velocity of an ionic substance and the applied electric field is given in eq. (1).

$$v_{\text{eff}} = m_{\text{eff}} \cdot E \quad (1)$$

$v_{\text{eff}}$  = effective velocity of an ionic substance under experimental conditions [ $\text{m} \cdot \text{s}^{-1}$ ].

$m_{\text{eff}}$  = effective mobility of an ionic substance under experimental conditions [ $\text{m}^2 \cdot \text{v}^{-1} \cdot \text{s}^{-1}$ ].

$E$  = applied electric field strength [ $\text{V} \cdot \text{m}^{-1}$ ].

In general  $m_{\text{eff}}$  of a particular ionic substance depends on the ionic radius and charge of the substance, viscosity of the solvent, and is a complex function of dissociation, complexation and solvation. An expression for  $m_{\text{eff}}$  is given in eq. (2):

$$m_{\text{eff}} = \sum_i m_i \cdot \alpha_i \cdot \gamma_i \quad (2)$$

$m_i$  = absolute ionic mobility

$\alpha_i$  = degree of dissociation

$\gamma_i$  = correction factor for retardation and relaxation effects.

So  $m_{\text{eff}}$  may be manipulated by controlling the pH, type of solvent and type and concentration of complexation agents. In fact  $m_{\text{eff}}$  determines to a large extent the resolution of an electrophoretic separation system. By manipulation of the physical/chemical conditions of the solution in which the sample is dissolved, charged substances may be separated. Furthermore, neutral substances which can be charged under the proper experimental conditions may be separated.

Electrophoretic sample pretreatment procedures discriminate between the charged and the neutral part of the sample. Moreover, these methods allow the separation between cationic and anionic substances and, to a certain extent, between similar charged substances of the sample [23, 24].

The appeal of electrophoresis as sample pretreatment techniques may also be due to the relatively simple equipment and, moreover, the promising possibilities of automation. Among the many electrophoretic techniques, zone-electrophoresis (ZE) and isotachopheresis (ITP) are of interest as sample pretreatment procedures [25, 26].

As far as ITP is concerned, there are two additional advantages with respect to other electrophoretic techniques,

i.e. the self-correcting property and the concentrating effect of the separated zones in the ITP process [23, 24].

In an ITP experiment, in for instance an anionic separation, three different electrolytes placed in three different parts of the equipment have to be distinguished, i.e. (Fig. 1)

- leading electrolyte, which contains the anion, i.e. leading ion  $L^-$ , with the highest effective mobility  $m_{L^-}$ , filling both the separation and leading electrolyte compartment.
- terminating electrolyte, which contains the anion, i.e. terminating ion  $T^-$ , with the lowest effective mobility  $m_{T^-}$ , filling the terminating electrolyte compartment.
- sample, which contains the anions, i.e.  $A^-$ ,  $B^-$ , etc., to be separated with effective mobilities;

$$(m_L)_{\text{eff}} > (m_{A^-})_{\text{eff}}, (m_{B^-})_{\text{eff}} > m_{T^-}$$

and which is placed in the sample compartment between leading and terminating electrolyte.

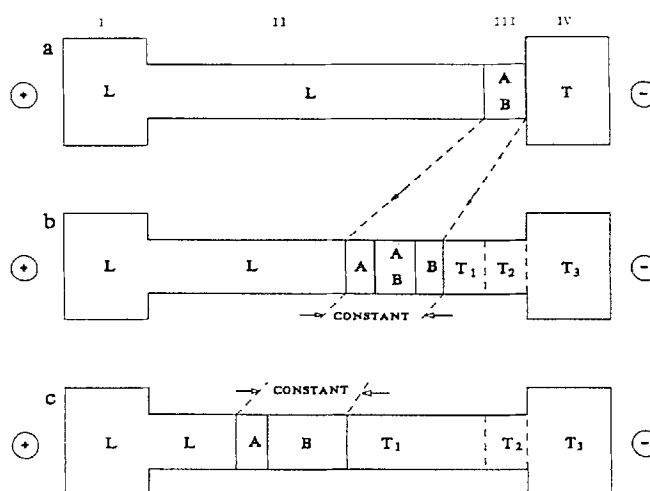


Fig. 1a

Separation of a mixture of anions according to the isotachopheretic principle. Sample A + B is introduced between the leading anionic species L and the terminating anionic species T.

Suitable cationic species are used as the buffering counter ion. The starting conditions are shown in (a). After a certain time (b) a mixed zone (AB) is obtained according to the moving boundary principle. Finally (c), all anionic substances of the sample are separated.

I, leading electrolyte compartment; II, separation compartment; III, sample introduction compartment; IV, terminating electrolyte compartment.

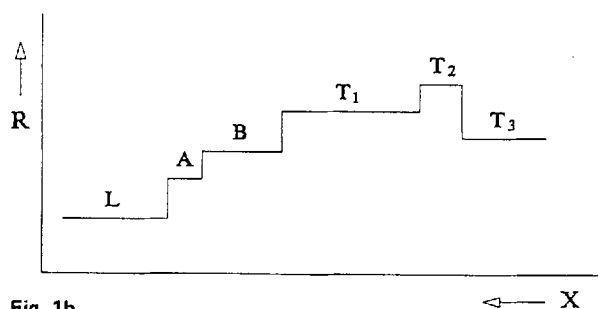


Fig. 1b

Graphical representation of the physical property, R, e.g. conductivity, temperature, as a function of the position x, in the separation equipment.

In this example the anionic species from the sample will start to migrate, when an electric field is applied, with effective velocities ( $v_{\text{eff}}$ ). The leading ions will migrate in front and are followed by respectively the anions of the sample and the terminating ion. The components will be separated by a moving boundary process. Since electro-neutrality has to be fulfilled, the separand zones will migrate with equal velocity ( $v_{\text{iso}}$ ), which is the isotachophoretic condition. Consequently each zone has its own electric field strength and conductivity.

After a certain time the sample will be separated in zones sandwiched between leading and terminating electrolyte as is schematically outlined in Fig. 1. So in analytical ITP the step heights along the vertical axis provide qualitative information (i.e. conductivity and potential gradient), while the lengths of the corresponding steps provide quantitative information.

As mentioned earlier, the self-correcting property and the concentrating effect are additional advantages of ITP over some other electrophoretic techniques. The self-correcting property of ITP corrects diffuse zone boundary, owing to i.e. diffusion, to sharp boundaries. This is due to the constant distinct field strengths in each zone after the steady state has been reached according to eq. (1).

In ITP the concentrating of each separand in its zone is given by the Kohlrausch regulating function [23, 24], i.e. for an anionic system (ion  $A^-$ ) the following equation can be derived:

$$C_{A^-} = C_L \cdot \frac{(m_L + m_P)}{(m_A + m_P)} \cdot \frac{m_A}{m_L} \quad (3)$$

$C_{A^-}$  = concentration of anion  $A^-$  of the sample in the separated zone.

$C_L$  = concentration of the leading ion.

$m_A$ ,  $m_L$  and  $m_P$  are the absolute mobilities of anion  $A^-$ ,  $L^-$  and counterion  $P^+$ , respectively.

From the Kohlrausch equation it follows that after reaching the steady state the concentration in each zone is constant and is determined by the composition of the leading electrolyte. So diluted samples will be concentrated according to (3) and more concentrated samples will be diluted.

Because of its increased sample capacity compared to analytical ITP, preparative ITP was applied in this study as a sample pretreatment technique.

Blank samples and spiked serum and urine samples of some drugs were subjected to preparative ITP on gel slabs of several compositions. The position of the specific zones in which the components of interest were concentrated, were indicated by coloured markers with mobilities similar to the components under study. This facilitated the cutting out and subsequent desorption of the components from the gel slab. The desorbates were evaporated to a certain extent to facilitate detection of the components.

Subsequently, the desorbates were analyzed by HPLC techniques. From the chromatograms the recoveries of the drugs from blank and spiked samples of body fluids were calculated.

## Experimental

### Analytical Isotachopheresis

The analytical capillary ITP experiments were performed in a home-constructed equipment which included a conductivity detector as described by Everaerts et al. [23]. The constant driving electric current was delivered by a Brandenburg type 807R power supply (Brandenburg, Thornton Heath, England).

The detector output was recorded with a potentiometric recorder, type BD 41 (Kipp & Zonen, Delft, The Netherlands).

The several operational systems, including typical leading and terminating electrolyte combinations, which were applied are listed in Table I. For anionic separations 0,2% w/w HEC (hydroxyethylcellulose) was added to the leading electrolyte in order to suppress electro-osmosis.

### Preparative Electrophoresis

Preparative ITP experiments were carried out on a LKB Multiphor II Electrophoresis unit equipped with an LKB 2197 Constant Power Supply with maximum current, voltage and power limits of 250 mA, 2500 V and 100 W respectively (Pharmacia LKB Biotechnology, Uppsala, Sweden). The preparative gel experiments were performed in two home-made separation compartments (Fig. 2), which could be positioned in the Multiphor II. These compartments allowed the testing of different electrophoretic carriers under otherwise identical experimental conditions.

The connections between the separation compartments and the leading and terminating electrolyte reservoirs were performed with flat sponges. To suppress the interferences by electro-osmosis, in a number of cases 0,2% w/w of the additives HEC (hydroxyethylcellulose) or MHEC (methyl-

**Table I.** Operational systems for isotachopheresis; solvent; water. Preparative ITP: current ca. 10–15 mA; voltage 600–1200 V; power 10 W limited. Capillary ITP: capillary tube, 0.4 mm i.d.; current ca. 70  $\mu$ A.

A	leading electrolyte	terminating electrolyte
Anion	$Cl^-$	MES
Concentration (Mol. $l^{-1}$ )	0.01	ca. 0.005
Counterion	histidine	histidine
pH	6.0	ca. 6.0
B	leading electrolyte	terminating electrolyte
Cation	$K^+$	$H^+$
Concentration (Mol. $l^{-1}$ )	0.01	0.005 HAc
Counterion	acetate	acetate
pH	5.0	3–4
C	leading electrolyte	terminating electrolyte
Anion	$Cl^-$	$\alpha$ -alanine
Concentration (Mol. $l^{-1}$ )	0.01	0.01
Counterion	ammediol	$Ba^{++}$
pH	8.9	ca. 10–11

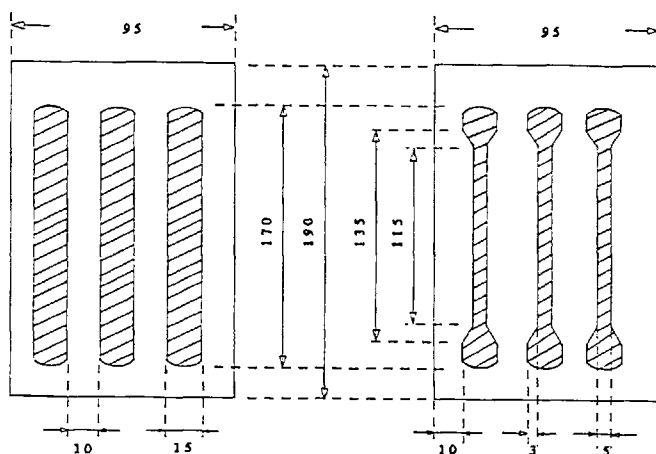


Fig. 2

Two home-constructed separation compartments for the preparative ITP experiments; dimensions in mm, construction material: Perspex.

Table II. Materials tested as electrophoretic carriers

Name	Structure	Manufacturer
Agarose	Galactopyranose polymer	Merck, Darmstadt, GFR
Celluloseacetate	Cellulose-polyacetate	Sepraphore III, Gelman Instrument, Ann Arbor, MI, USA
Sephadex	Glucose polymer	Pharmacia, LKB, Uppsala, Sweden
Ultrodex	Glucose polymer, modified	Pharmacia, LKB, Uppsala, Sweden
Glass beads	silicon dioxide 140–160; 180–200 $\mu\text{m}$	Applied Science Lab. Inc.

hydroxyethylcellulose) was dissolved in the leading electrolytes. The materials that have been tested as carriers for preparative electrophoresis are listed in Table II. The gel slabs were prepared by slurring the dry materials in water in a suitable ratio (except for agarose which had to be dissolved at 100°C and cooled to 70°C) and then pouring out into the separation compartment. The cellulose acetate sheets could be used with no preparation other than soaking them in the various electrolytes.

To trace the zones of interest in the preparative ITP step, coloured markers with mobilities comparable to the test components were added to the samples and separated under the experimental conditions. The markers and test components, including the relative step heights relative towards the terminating electrolyte, are listed in Table III.

After the completion of the separation the part of the gel slab in which the coloured zones occurred, was cut out and transferred to an LKB 2117-502 desorption elution tube, equipped with a 10  $\mu\text{m}$  nylon frit. The desorption of the components from a zone was performed with three small amounts of methanol and/or water. In blank experiments

Table III. Relative step heights (RSH) of markers and test components relative to the terminating zone; see operational system Table I

Name	RSH (%)
Anions; operational system A	
amaranth red	24
congo red	36
bromophenol blue	52
phenylacetic acid	57
Cations; operational system B	
Methylene blue	35
neutral red	37
safranin O	46
malachite green	72
galanthamine	74
codeine	75
morphine	74
Anions; operational system C	
bromothymol blue	34
theophylline	37

it was determined that after consecutive elution with 3 portions of small volumes of the solvent, the components of interest were completely desorbed. This procedure was applied in all desorption procedures. The collected desorbates were analyzed by HPLC techniques.

### Liquid Chromatography

The HPLC analyses were performed on a Pharmacia LKB instrument consisting of a type 2150 pump and a variable wavelength UV-detector type 2151. Injections of the desorbate samples were made with a Rheodyne 7125 injector (Rheodyne Incorp., CA, USA), equipped with 20  $\mu\text{l}$  sample loop.

The detector output was recorded with a potentiometric recorder, type BD 40 (Kipp & Zonen, Delft, The Netherlands). Calculations of the chromatographic data were performed with a Spectra Physics SP 4000 integrator (Spectra Physics, CA, USA).

The different phase systems and other experimental conditions, applied for the HPLC analyses, are listed in Table IV.

### Chemicals

Theophylline, morphine, codeine, galanthamine and phenylacetic acid were used as relevant drugs or metabolites for recovery studies from blood, urine and blank samples.

All chemicals were of at least analytical grade and purchased from either Sigma (St. Louis, MO, USA) or Merck (Darmstadt, GFR).

### Results and Discussion

The materials listed in Table II were tested as electrophoretic carriers for either anionic and cationic substances. Electrophoretic carriers for preparative applications should meet two criteria:

- i. they must allow the introduction of a finite amount of sample;
- ii. the carriers must not show strong adsorption effects towards sample components which may result in diffuse zones.

This latter is particularly important with respect to a selective and complete desorption of the components from the carrier.

Small amounts of the anionic and cationic markers, as listed in Table III, were introduced to the different gel slabs and the cellulose acetate sheet. Subsequently, they were subjected to an ITP separation process with the corresponding operational system. The criterion of whether the tested carriers were suitable for these purposes or not, was the occurrence of sharp zones. From the results listed in Table V, it can be concluded that under the experimental conditions Ultradox can be applied for cationic and anionic substances; glass beads and celluloseacetate are only suitable for anionic separations, while Sephadex can be used for cationic separations.

The explanation of the observed unsharpness of the zones in some cases, indicating strong interactions between sample substances and the carrier, was not the first aim of this study. However, in the case of the cation separations with glass beads, the relative unsharpness of the zones can be explained by the adsorption of the cations by the silanol groups on the glass beads.

In general the surface of carrier materials, for instance glass beads, are negatively charged due to either desorption of

**Table IV.** Experimental conditions of HPLC analysis of the desorbates

A. Column:	length 13 cm; 4.6 mm i.d.; 5 $\mu$ m C-18 modified silica (Brownlee labs., USA)
Eluent:	water-methanol 60 : 40 v/v
Detection:	UV- 280 nm
Flow:	1 ml/min.
B. Column:	length 13 cm; 4.6 mm i.d.; 5 $\mu$ m C-18 modified silica (Brownlee labs., USA)
Eluent:	methanol-phosphate buffer pH = 3.0 in water, 30 : 70 v/v
Detection:	UV- 240 nm
Flow:	1 ml/min.
C. Column:	length 10 cm; 8 mm i.d.; 4 $\mu$ m Novapak C-18 (Millipore Waters, USA)
Eluent:	acetonitrile-pentanesulfonic acid buffer, pH = 2,0 in water, 13 : 87 v/v as described by Tebbet et al. [27]
Detection:	UV- 235 nm
Flow:	2.0 or 2.5 ml/min

**Table V.** Results of the test of several materials as a carrier for preparative isotachopheresis: + = sharp zones; - = unsharp, diffuse zones,  $\circ$  = not investigated

Carrier	Anions	Cations
Glass beads	+	-
Agarose	$\circ$	-
Ultradox	+	+
Sephadex	$\circ$	+
Celluloseacetate	+	-

cations or adsorption of negatively charged compounds. The liquid near the surface is positively charged, i. e. zeta-potential, and consequently an electric double layer is formed. The surface charge also depends on, for example, the pH and ionic strength of the solution. The results are twofold, in the first place cationic constituents are much easier adsorbed. Secondly there is an electro-osmotic flow towards the anode, which is proportional to the zeta-potential and dielectric constant of the liquid and inversely proportional to the viscosity. The disturbance due to electro-osmosis can be suppressed by addition of high viscous substances, such as HEC and MHEC.

For further investigations we selected Ultradox for cationic and glass beads for anionic separations.

Subsequently blank aqueous solutions of several amounts of theophylline, phenylacetic acid, morphine, galanthamine and codeine were subjected to preparative ITP in order to study the recoveries of these components from the electrophoretic carriers, Ultradox for cations and glass beads for anions, under the experimental conditions.

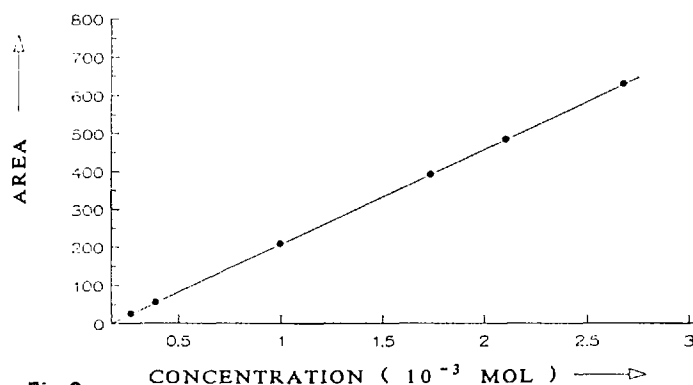
After the ITP-experiments and subsequent desorption of the components from the specific zones, quantitative analyses were performed by one of the HPLC methods. For the quantitative HPLC analysis standard curves of the

**Table VI.** Blank recovery experiments of theophylline, standard solution 4.0 mM in water; ITP-carrier, glass beads; marker, bromothymol blue, ITP system A; HPLC system A

Extractant	Medium (0.2%)	Amount [nmol]	Recovery (%)
Methanol	MHEC	30	88
Methanol	MHEC	30	87
Methanol	MHEC	10	85
Methanol	MHEC	20	87
Water	MHEC	2	90
Water	MHEC	5	93
Water	MHEC	10	98
Methanol	HEC	5	90
Methanol	HEC	10	83
Water	HEC	10	82
Water	HEC	5	82

Mean value 88%

SD (abs) 5%



**Fig. 3** Standard curve for the HPLC analysis of phenylacetic acid. HPLC system B; measuring data ( $\bullet$ ); coefficient of correlation 0.998.

different test components were made under the relevant experimental conditions. The curves showed a satisfying correlation of at least 0.998. An example, for phenylacetic acid, is given in Fig. 3. In Tables VI to VIII the results of the recovery experiments are summarized including the applied preparative ITP and the HPLC system. From these

**Table VII.** Blank recovery experiments of phenylacetic acid, standard solution 2.0 mM in water; ITP-carrier, glass beads; marker, bromophenol blue, ITP system A; desorbating liquid, methanol; HPLC system B

Medium	Amount [nmol]	recovery (%)
MHEC	200	94
MHEC	400	94
MHEC	200	89
MHEC	400	91
HEC	100	83
HEC	400	90
HEC	100	89
HEC	200	89
HEC	400	88
HEC	100	90
HEC	200	87
HEC	400	91

mean value 90 %  
SD (abs) 3 %

**Table VIII.** Blank recovery experiments from aqueous solutions of morphine HCl, standard 22.3 mg/l; galanthamine HBr, standard 20.7 mg/l and codeine HCl, standard 44.0 mg/l; injected volumes on ITP-slabs, 100  $\mu$ l; ITP-carrier, ultradex; markers malachite green and methylene blue; ITP system B; HPLC system C

Morphine (%)	Galanthamine (%)	Codeine (%)
90	90	89
90	90	83
90	85	84
90	88	87
90	89	89
91	83	88
90	91	89
91	89	89
87	84	84
90	88	87
1	3	2

mean value  
SD (abs)

data it can be concluded that the recoveries of the components under study from aqueous solutions are in between 85–90% with a standard deviation of 1–5%. In practice the manual cutting out of the zones causes a loss of some of the gel of the zones of interest. Therefore it is probable that the recoveries are 10–15% lower as could be expected. This may significantly improved by cutting out the zones more quantitatively through instrumental improvements and/or on-line ITP-HPLC coupling. Nevertheless, within the instrumental limitations of this off-line ITP procedure, these recoveries are satisfactory and reproducible.

Next, urine and serum samples were spiked with several amounts of morphine, codeine and galanthamine. Subsequently these samples were subjected to an ITP-pre-treatment procedure. After desorption of the components in the specific zones, the desorbates were analyzed by HPLC. The results of these experiments are summarized in Table IX.

The data of these recovery experiments of serum and urine samples show in general a significant decrease towards the recoveries from aqueous samples. Moreover, the recoveries tend to decrease at lower amounts of the spiked alkaloids in serum and urine samples. These observations may be explained by a hindered electrodesorption process from the samples. Urine and blood contain an amount of ionic constituents, which cause a relatively high ionic strength in these samples. After the introduction of such samples, at the beginning of the ITP separation, a decreased electric field strength will occur over the sample zone, due to the relatively high conductance of this zone. Therefore, a certain amount of the components of interest may be retarded in the terminating electrolyte during the moving boundary state, which anticipates the steady state of the ITP process. In spite of the self-correction of ITP, these retarded components will not reach the different zones, during the separation time. These interferences and consequent loss of components from the sample will be more significant at lower concentration of sample components.

The standard deviations of 2–4% observed in these experiments indicate that the method is of the same reproducibility as in the experiments with aqueous solutions.

Besides the above mentioned effects, the recovered amounts of some substances may also be decreased due to protein binding in these in-vitro experiments. If the substances are reversibly bonded and the rate of dissociation is high in

**Table IX.** Recovery experiments of morphine HCl, galanthamine HBr and codeine HCl from aqueous solutions (a), spiked serum (b) and spiked urine (c). ITP-carrier: Ultradex; markers: methylene blue and malachite green; ITP system: B; HPLC system: C; n = number of experiments

Sample	n	Injected vol. ( $\mu$ l)	Concentration (mg. $l^{-1}$ )			Recoveries (%)		
			Morphine	Galanthamine	Codeine	Morphine	Galanthamine	Codeine
a	9	100	22.3	20.7	44	90 $\pm$ 1	88 $\pm$ 3	87 $\pm$ 2
b	5	100	22.3	20.7	44	83 $\pm$ 3	81 $\pm$ 2	83 $\pm$ 2
b	6	500	2.23	2.07	4.4	74 $\pm$ 2	70 $\pm$ 3	79 $\pm$ 2
b	6	500	0.223	0.207	0.44	76 $\pm$ 4	77 $\pm$ 4	76 $\pm$ 2
c	8	100	22.3	20.7	44	69 $\pm$ 3	75 $\pm$ 2	73 $\pm$ 2
c	5	500	0.223	0.207	0.44	76 $\pm$ 3	41 $\pm$ 2	67 $\pm$ 4

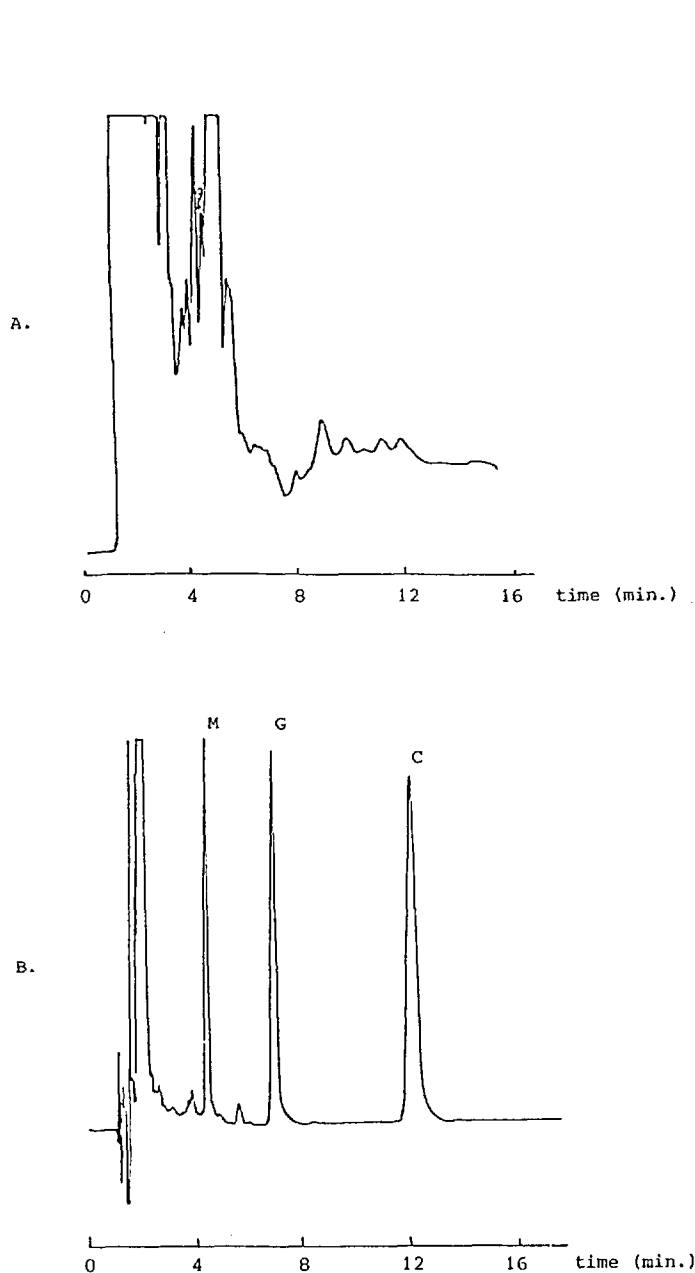
comparison with the electrophoretic process, total recovery of the substances can be achieved. In other cases the recovery depends on the operational and sampling conditions. In an electric field the protein binding may be broken which makes desorption of even strongly bonded substances possible.

An additional advantage of ITP sample pretreatment is the favourable influence on the HPLC columns used for the analysis. We observed in our laboratory a considerably extended lifetime and chromatographic stability when the ITP pretreatment was applied. Some examples of the powerful clean-up effects of the ITP pretreatment procedure are presented in Figs. 4 and 5 for a serum and urine sample, respectively.

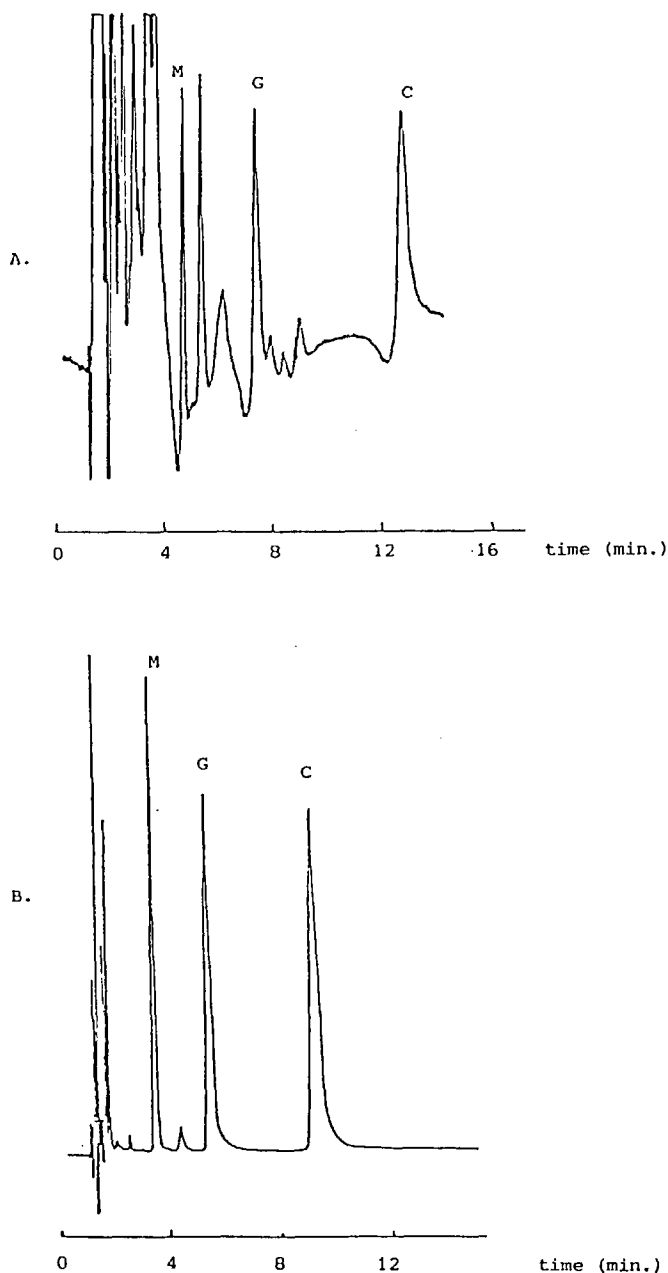
Comparisons of HPLC analysis of ultrafiltrated spiked serum samples and spiked serum samples, subjected to the ITP pretreatment indicate that a significant part of the protein bindings is broken.

The overall detection limits of the presented methods for galanthamine and morphine are 10 ng/ml. For codeine this values is 20 ng/ml. The reagents applied in the preparative ITP step (markers, leading and terminating electrolytes), did not interfere with the HPLC separations as far as we are concerned.

In conclusion, preparative ITP offers attractive possibilities as a sample pretreatment technique prior to HPLC analysis. Nevertheless more research should be spent on the problems



**Fig. 4**  
HPLC chromatograms of morphine (M), galanthamine (G) and codeine (C) in a filtrated (A) and ITP-pretreated (B) urine sample. ITP system B; HPLC system C.



**Fig. 5**  
HPLC chromatogram of morphine (22.3 mg/l) [M], galanthamine (20.7 mg/l) [G] and codeine (46.0 mg/l) [C] in an untreated, ultrafiltrated (A) and ITP pretreated (B) serum sample. ITP system B; HPLC system C.



of the electro-desorption at low drug concentration in samples with high ionic strength and/or strong protein binding properties.

## Acknowledgement

The authors gratefully acknowledge Pharmacia LKB Biotechnology Division, Woerden, NL for putting the preparative electrophoretic equipment to our disposal.

We thank Mr. M. J. S. van Thiel for fruitful discussions and technical support and Mrs. D. Tjallema for the accurate handling of the manuscript.

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Received: Sept. 27, 1988  
Accepted: Dec. 19, 1988  
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