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Sample matrix effects in capillary electrophoresis

II. Acetonitrile deproteinization

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ABSTRACT

Acetonitrile deproteinization [acetonitrile–serum (3:2, v/v)] was found to be a suitable method for removal of serum proteins for the analysis of drugs and small molecules by capillary electrophoresis. Many compounds exhibited higher plate numbers and peak heights in the presence of 60% acetonitrile. This effect is due to a special stacking brought along by the low resistivity of the acetonitrile in the sample. In addition to removing serum proteins, acetonitrile improves the solubility of some compounds and increases the sample volume which can be introduced into the capillary. Serum theophylline analysis was used here as an example for the analysis of drugs by capillary electrophoresis using acetonitrile deproteinization.

INTRODUCTION

The sample matrix plays an important role in capillary electrophoresis. Under proper conditions a stacking effect [1–4] can be obtained by preparing the sample in a buffer of ten times lower ionic strength than the running buffer leading to sharper peaks, higher plate number [1], and an increased detector signal [2]. In the previous work, it was shown that some drugs and metabolites can be determined in a complex biological matrix, such as serum, directly by dilution [5,6], especially if a high ionic strength running buffer is used. However, for analytes present in low concentrations, the ions and proteins present in serum interfere with their assay. The majority of drugs are present in very low concentrations in serum. Proteins may interfere by three means: by binding the drug, masking its absorption, and adsorbing to the capillary walls affecting reproducibility. In order to detect these compounds by capillary electrophoresis (CE) a clean-up step is necessary. Although liquid- and solid-phase extraction are excellent means for extraction and concentration of drugs and small molecules, these methods are time consuming and therefore they are not suitable for rapid assays such as demanded in clinical laboratories [7]. A simple alternative technique which has been used successfully in HPLC is acetonitrile deproteinization [5]. Here, we optimize this method for CE and show that this method has some advantages. In addition to removing proteins, it can increase the plate number and peak height for some compounds by a special stacking effect.

MATERIALS AND METHODS

Instrumental

An automated capillary electrophoresis instrument (Beckman, Palo Alto, CA, USA) was set at 13 kV, 24°C and 254 nm. The capillary was 25 cm × 50 μm (I.D.). The electrophoresis buffer was 300 mM boric acid adjusted to pH 8.5 with sodium hydroxide. Samples were introduced by pressure injection for 15 s. The capillary was washed for 1 min with the electrophoresis buffer after each sample.

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Samples

As a model compound, we used in most of the experiments iohexol {bis(2,3-dihydroxypropyl)-5-[N-(2,3 dihydroxypropyl)-acetamido]-2,4,6-tri-iodoisophthalamide} (Winthrop Pharmaceuticals, New York, NY, USA), a tri-iodinated, non-ionic radiographic contrast medium which is a highly water-soluble compound and does not bind to serum proteins.

Serum deproteinization

Serum (100 μl) was deproteinized by mixing with 150 μl acetonitrile for 15 s and the mixture was centrifuged for 1 min at 15 000 g.

Plate number (N)

Calculation [1] was based on the electrophoretic mobility of the compound (t_R) and width of the peak at half height ($W_{1/2}$) using the formula $N = 5.5(t_R/W_{1/2})^2$.

Serum theophylline analysis

The same method of serum deproteinization was used except the acetonitrile contained 25 mg/l of 3-isobutyl-1-methylxanthine (Aldrich, Milwaukee, WI, USA) as an internal standard. The supernatant was pressure injected for 6 s and electrophoresed at 11 kV with detection at 280 nm using a capillary of 25 cm × 75 μm.

RESULTS AND DISCUSSION

Acetonitrile is a very good deproteinization agent. However, in capillary electrophoresis, it does not conduct current. Mixtures of acetonitrile in aqueous solutions on the other hand, do conduct current. The effect of 60% acetonitrile compared to that of water on the separation of several compounds is illustrated in Fig. 1. The separation is improved; the peak height is higher and the retention time is increased. However, the extent of these effects differ from one compound to another. The increase in peak height for iohexol (about five fold) is much greater than that for the other compounds. This improvement in peak height and plate number was also present and further enhanced if these compounds were added to the supernatant of serum deproteinized with acetonitrile (final acetonitrile

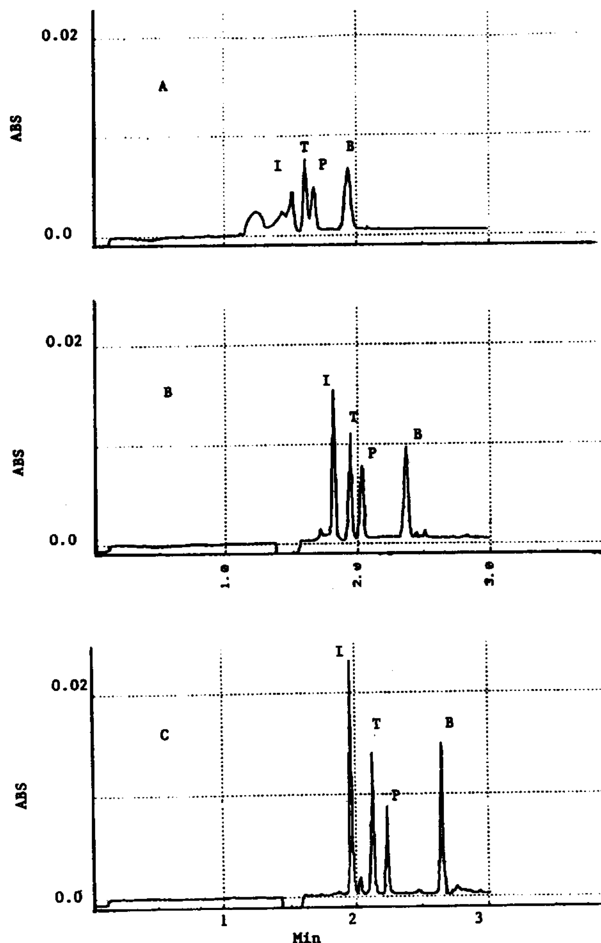

Fig. 1. Electropherogram of different compounds dissolved in: (A) water, (B) acetonitrile–water (60:40), and (C) supernatant of serum deproteinized by acetonitrile. I = iohexol, T = theophylline, P = phenytoin, B = phenobarbital. Capillary 25 cm × 50 μm, 15-s injections, 13 kV. ABS = absorbance in AU.

concentration 60%). Thus acetonitrile does not just remove serum proteins but also improves the peak height of some compounds.

Since the major ions in serum are sodium and chloride at about 150 mM an equivalent amount of NaCl was added to the acetonitrile mixture. Further improvement in peak height and plate number occurred following NaCl addition (Table I). Different alcohols which occasionally are used for deproteinization also increased the plate number but not to the extent of that of acetonitrile (Table II) The acetonitrile effect on plate number and peak height occurs with large sam-

TABLE I

EFFECT OF DIFFERENT ACETONITRILE CONCENTRATIONS ON PEAK HEIGHT (mAU) AND PLATE NUMBER FOR IOHEXOL (200 mg/l)

Sample introduced by pressure injection for 15 s.

Sample diluent	$N \cdot 10^3$	Absorbance (mAU)
Serum deproteinized by acetonitrile (60%)	110	44
In absence of 60 mM NaCl in the sample		
30 m*M* borate	35	15
300 m*M* borate	10	6
% Acetonitrile in water		
0	21	11
10	27	13
20	32	14
40	37	18
60	59	26
In presence of 60 mM NaCl in the sample		
% Acetonitrile		
0	35	20
10	37	22
20	48	37
40	108	42
60	120	44

TABLE II

EFFECT OF DIFFERENT ALCOHOLS IN THE PRESENCE OF 60 m*M* NaCl ON IOHEXOL (200 mg/l) PEAK HEIGHT (mAU) AND PLATE NUMBER

Alcohols used for deproteinization	$N \cdot 10^3$	mAU
Water	35	19
60% Acetonitrile	120	42
60% Methanol	30	21
60% Ethanol	40	26
60% 1-Propanol	52	27

ple volume (Table III). The high sample volumes used here represent capillary overloading. Thus, it seems that acetonitrile increases the capacity of the capillary for a larger sample volume as it is common in stacking. The stacking effect increases at lower voltages approaching a plate number close to 400 000 for the 25-cm capillary (Table IV).

The stacking effect produced here by the acetonitrile shares some similarities, as well as some differences, with the traditional stacking produced in low concentration of aqueous buffers. In both cases, the stacking is produced by the difference in the resistivity of the sample and the running buffer and it allows a larger sample volume to be introduced into the capillary. However, the stacking in acetonitrile is different because the high resistivity of the sample results here from introduction of an organic solvent into the sample. In addition, to removing the proteins, acetonitrile improves the solubility of some compounds but not all. For example phenobarbital is more soluble in acetonitrile than in water. On the other hand iohexol which exhibits here a better stacking effect than phenobarbital is a

TABLE III

EFFECT OF SAMPLE VOLUME BY PRESSURE INJECTION (s) AND ACETONITRILE ON PEAK HEIGHT (mAU) AND PLATE NUMBER

Sample volume (s)	60% Acetonitrile				0% Acetonitrile			
	+NaCl		−NaCl		+NaCl		−NaCl	
	$N \cdot 10^3$	mAU	$N \cdot 10^3$	mAU	$N \cdot 10^3$	mAU	$N \cdot 10^3$	mAU
20	110	40	45	40	–	–	–	–
15	115	30	75	31	26	13	15	11
10	120	21	65	24	41	13	40	11
5	102	10	64	12	101	10	96	11
3	95	5	30	5	122	6	115	7
1	90	2	–	–	110	2	–	–

TABLE IV

EFFECT OF THE VOLTAGE (kV) ON PLATE NUMBER

Sample introduced for 10 s.

kV	$N \cdot 10^3$	
	60% Acetonitrile (+NaCl)	0% Acetonitrile (−NaCl)
16	96	37
14	140	48
10	172	58
6	314	138
3	395	159

highly water-soluble compound (>300 mg/ml). The stacking effect is also aided by the presence of relatively high ion concentrations in the sample (optimum about 100 m*M* for NaCl) which may improve the stacking by forming a transient isotachophoresis or interacting with the capillaries walls. The effect of acetonitrile on stacking is more pronounced in short-length capillaries (about 25 cm) compared to longer ones. Although we previously [6] used acetonitrile to deproteinize serum samples for CE, its stacking effect was not well appreciated.

The main purpose in using acetonitrile is to remove serum proteins. In HPLC, a 50% ratio of acetonitrile to serum has been used traditionally. Fig. 2 shows that this ratio is not adequate to remove serum proteins, especially albumin. In HPLC, albumin binds to the column packing and does not appear on the chromatogram. In CE, albumin migrates and can be seen on the electropherograms. Increasing the acetonitrile ratio to 60% was more effective in removing serum proteins.

To illustrate the suitability of this technique for analyzing drugs in serum, we measured serum theophylline by this technique. The regression analysis for serum theophylline assayed by this method compared to a fluorescence immunoassay is good (CE = 0.98immuno − 4, $n = 21$, $r = 0.98$). The test was linear between 3–40 mg/l. Fig. 3 shows an electropherogram from a patient on theophylline treatment. In order to avoid the variable effects of different ions on separation and drug recovery, the standards were prepared in serum matrix rather than in aqueous solutions.

These data show that acetonitrile extraction is

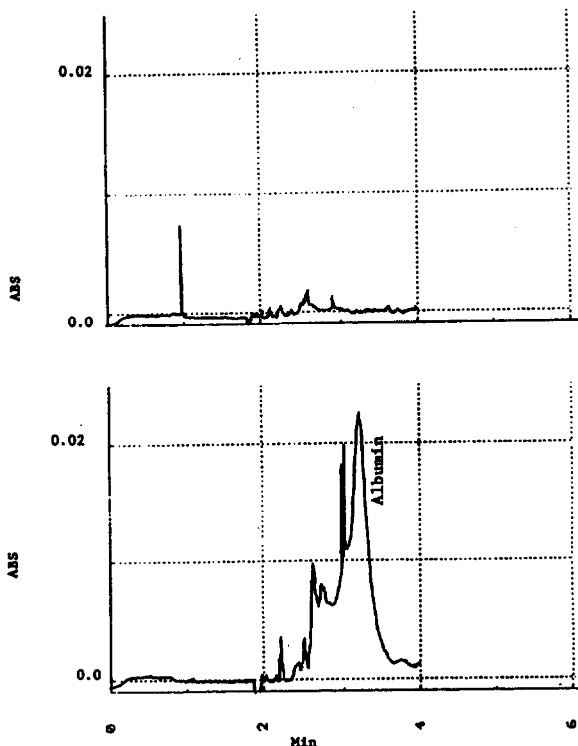

Fig. 2. Electropherogram of serum supernatant using: (top) 60% deproteinization and (bottom) 50% deproteinization. Capillary 25 cm × 50 μm, 10-s injections, 13 kV, 214 nm wavelength. ABS = absorbance in AU.

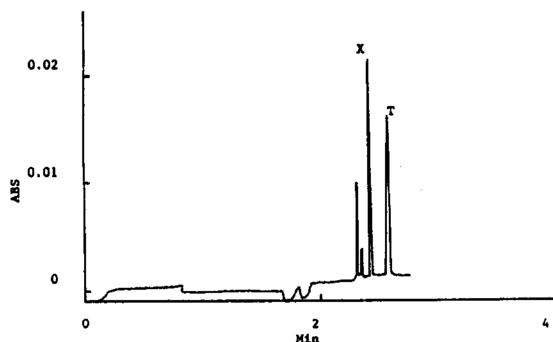

Fig. 3. Electropherogram of serum theophylline (10 mg/l) from a patient treated with the drug. T = theophylline, X = internal standard = 3-isobutyl-1-methylxanthine. Capillary 25 cm × 75 μm, injection 6 s, 11 kV. ABS = absorbance in AU.

a good method for preparing samples for CE. This method permits the introduction of larger volumes into the capillary with an increase in plate number for many compounds. Previously, we have shown that reproducibility of the assay is improved when acetonitrile is used compared to serum dilution [5] and furthermore, the capillary does not need extra washing with sodium hydroxide between samples leading to a faster analysis time. This work demonstrates that CE has a good potential for therapeutic drug monitoring especially for those compounds lacking a good immunoassay.

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