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Concentration and separation of hypoglycemic drugs using solid-phase extraction–capillary electrophoresis

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Abstract

Solid-phase extraction–capillary electrophoresis (SPE–CE) is a technique whereby very dilute analytes may be selectively extracted from a sample matrix and concentrated on-line for analysis. This study describes the first phase in the development of a method exploiting this technique for the direct analysis of hypoglycemic drugs in urine. Effective separation and detection of six sulfonylurea drug standards at concentrations below the detection limit of conventional capillary electrophoretic techniques is shown to be attainable. Since surfactant interfered with the on-line concentration process, non-MEKC (micellar electrokinetic chromatography) separation conditions were defined. Using 250 m*M* borate/5 m*M* phosphate at pH 8.4, all drugs in a mixture at 285 ng/ml were effectively extracted, concentrated from an injected volume of 2.5 μl, non-selectively desorbed with an organic-based elution buffer and electrophoretically resolved. Sample loading was found to be linear in the 0.12–1.9 μl range and drugs in a volume of up to 190 μl could be concentrated and detected with a sensitivity of $\approx$5 ng/ml. Not only was resolution of the desorbed material uncompromised by the presence of the SPE-tip, but separation of glipizide and glyburide was observed despite the fact that these drugs were unresolved under the same separation conditions by standard capillary zone electrophoresis (CZE). From these results, it is clear that SPE–CE not only increases the sensitivity for detection but that selectivity may be altered due to chromatographic processes occurring on the solid-phase resin.

1. Introduction

Capillary electrophoresis (CE) has been shown to be useful for a diverse array of molecules including ions [1], sugars [2,3], peptides [4,5], oligonucleotides [6] and proteins [7,8]. Of particular relevance to the present study, the free solution capillary zero electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) modes have proven to be extremely useful for the analysis of a variety of drugs [9–15]. This has revealed the potential for CE in the clinical laboratory [16] and, in particular, for the analysis of drugs in body fluids [17].

Sulfonylurea drugs such as glipizide (Gp), glyburide (Gb), chlorpropamide (Cp), acetohexamide (Ah), tolbutamide (Tb) and tolazamide (Ta) have been utilized extensively for the treatment of hyperglycemia. "Factitious" or "drug-

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induced" hypoglycemia has been reported as a result of the surreptitious use of sulfonylurea drugs. Problems associated with their surreptitious abuse arise from the differential diagnosis of drug-induced hypoglycemia, which is difficult to distinguish from insulinoma (pancreatic tumor)-induced hypoglycemia [18] and has actually led to unnecessary exploratory surgery and even partial removal of the pancreas (subtotal pancreatectomy) [19]. At the present time, there are relatively few effective analytical methodologies for detecting sulfonylurea drugs in biological fluids. Although semi-quantitative HPLC methodology has been described [20] for detecting chlorpropamide in plasma at a concentration of 10 μg/ml, these levels are dramatically higher than the urinary levels expected with sulphonylurea drugs (100–500 ng/ml). GC–MS has proven to be problematic as a result of the non-volatility and difficulty in derivatizing the sulfonylurea drugs, while LC–MS is not commonly used. It is for these reasons that a capillary electrophoretic method for these compounds has been pursued [21]. MEKC was shown to provide rapid analysis for both second and third generation sulfonylurea drugs in less than 8 min. However, the extensive off-line extraction procedure required for partial purification of the urine prior to CE analysis makes it cumbersome and costly. Hence, it was clear that an on-line preconcentration of the drugs from urine would improve the cost-effectiveness of this assay.

On-line sensitivity enhancement with CE is attainable through a number of approaches including stacking [22] and isotachophoresis [23]. However, despite their effectiveness with many analyte systems, these preconcentration methods are often either unsuitable or lack the simplicity of a packed-inlet concentrator method such as that provided with SPE–CE. It is noteworthy that the preconcentration strategy employed with SPE–CE methodology is not novel. Guzman [24], Guzman et al. [25] and Fuchs and Merion [26] have described devices similar in construction and implementation to the SPE concentrator tip. Debets et al. [27] also employed a concentration "micro precolumn" (reversed-phase C_8) which was switched on- and off-line to a CZE capillary. This device allowed for the extraction and concentration of analytes from extremely large sample volumes, but required a liquid pump for sample loading and off-line switching to perform the CZE separation, all of which added to the complexity of the apparatus. Benson et al. [28] and Schwartz and Merion [14] have demonstrated the usefulness of a solid-phase (C_{18}) packed inlet capillaries for the analysis of drugs. Interestingly, Beattie et al. [29] independently developed a solid-phase concentrator virtually identical in construction and implementation to the SPE–CE concentrator tip described in this study. Beattie's results correlate well with our own observations in that concentrators constructed in this manner are robust, reusable and can reproducibly provide analyte concentration/separation that does not markedly deteriorate with normal use.

The SPE–CE concentrator described in the present study remains on-line and, with a short rinse to regenerate the reversed-phase, can be used for multiple consecutive analyses. Since there is no requirement for exotic instrumentation for detection and interpretation of results [30] or modification of the commercial P/ACE (Beckman Instruments) unit, the SPE–CE experiments can be performed under an automated, programmed sequence and allow for unattended operation and a high degree of reproducibility. These characteristics are perfectly suited to application of this technology for clinical method development.

In this study, we describe the results of the first phase of this study for implementing solid-phase extraction–capillary electrophoresis (SPE–CE) for the on-line analysis of sulfonylurea drugs. The ultimate goal of this approach, phase two, is to identify hypoglycemic drugs directly in urine without the requirement for a labor-intensive off-line solid-phase extraction procedure. This is difficult due to the micro-scalar nature of the concentrator but will most likely be achieved by exploiting the fine instrumental control capabilities of modern CE instrumentation for sample clean-up and selective elution. The results of the phase-one studies show the potential of SPE–CE for the analysis of hypoglycemic drugs at dilute

concentrations (ng/ml) below the detection limit of standard CZE. The performance of the SPE-tip is evaluated in terms of the loading capacity, sensitivity and linearity for concentrating and electrophoresing these particular drugs at low concentrations.

2. Experimental

2.1. Materials

Acetonitrile (ACN; HPLC-UV spectral grade) and hydrochloric acid (HCl) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Phosphate buffer (50 m*M*, pH 2.5) was purchased from Scientific Resources (Eatontown, NJ, USA). C_{18} material was removed from a SPE™ column purchased from JT Baker (Phillipsburg, NJ, USA). Glass fiber was removed from the bed support of a common disposable "spin column". Polyethylene tubing, IntraMedic #7405, was purchased from Clay Adams (Parsippany, NJ, USA). Poros resin (R20), was provided courtesy of PerSeptive Biosystems (Framingham, MA, USA).

2.2. CE instrumentation

HPCE separation was carried out on a Beckman P/ACE System 5510 equipped with a monochromatic UV detector. An IBM 486 ValuePoint computer utilizing System Gold software (V.8.1) was used for instrument control and data collection. All peak information (migration time and integrated peak areas) was obtained through the System Gold software.

2.3. SPE–CE capillary construction

The SPE capillary is a hybrid design consisting of two parts, a replaceable concentrator tip and a separation capillary. The concentrator tip for this study contains a 50–200 nl bed of reversed-phased (C_{18}) material that is dry-packed into a polyethylene sleeve between two frits (glass fibers, gently tamped into place) so that two short sections of capillary can be slid into the sleeve to retain the entire assembly and provide a tight seal without adhesives. The device is relatively resistant to blockage by the use of solid phase of a large sized mesh and the glass fiber frits forming the bed supports. The high flow-rate and minimal restriction allow the assembly to remain on-line with the separation capillary throughout the analysis and may remain on-line for multiple analysis if desired. Breakage of the tip or capillary joint and exposure to incompatible buffers are the most common events requiring assembly of a new unit. The complete assembly of the SPE–CE concentrator tip is usually accomplished in minutes with an uncoated separation capillary. The concentrator tip is mated to the inlet of the separation capillary (preinstalled in a P/ACE cartridge) by removing a short section of the inlet leg and replacing it with the SPE–CE concentrator tip. This is accomplished by joining the two sections with a second polyethylene sleeve which should be renewed every time an inlet section is replaced. The capillary tip used for the standard CZE separations is the same section of separation capillary that was originally removed to accommodate the SPE–CE concentrator tip. In this way the same separation capillary can be used both for SPE–CE or standard CZE separations dependent on the type of tip attached to the inlet.

2.4. CZE and SPE–CE separation methods

The capillary was rinsed with three column volumes of elution buffer, and then re-equilibrated with ten column volumes of separation buffer prior to sample injection. The CZE separations as well as sample concentration and release were as described in the figure legends. The CZE and SPE–CE capillaries were rinsed with elution buffer (0.5 min) as a post-separation treatment for regeneration of the solid phase. For SPE–CE analyses, standard drug mixtures were prepared by diluting stock solutions in 100% methanol into water.

2.5. Evaluation of SPE-tip performance

Lifetime

A newly assembled SPE-capillary with a 0.5 mm × 370 μm packed bed of C_{18} reversed-phase and a 47 cm × 75 μm I.D. (40 cm to detector) separation capillary was used to test the performance of the tip for no fewer than 30 consecutive separations. The reversed-phase material was regenerated with a short (0.5 min at $1.38 \cdot 10^5$ Pa) rinse using ACN–separation buffer (80:20) for regeneration of the phase between analysis.

Sample loading capacity

Total sample capacity of the 0.5 mm × 370 μm SPE concentrator was determined by doubling the injection time over a range of 0.5–32.0 min with an applied injection pressure of $3.4 \cdot 10^3$ Pa. The concentration of the Gp and internal standard (I.S.) used for these experiments was 2 μg/ml. Integrated peak areas were obtained using the System Gold software.

Detection sensitivity

A mixture containing glipizide and the internal standard was used as a means of determining the detection sensitivity of SPE–CE using a standard SPE-tip and UV detection at 200 nm. A simple two-component mixture was used for this study and contained Gp and I.S. at 2 μg/ml (0.2 min at $1.38 \cdot 10^5$ Pa; 1.9 μl), 200 ng/ml (2.0 min at $1.38 \cdot 10^5$ Pa; 19 μl) and 20 ng/ml (20 min at $1.38 \cdot 10^5$ Pa; 190 μl).

3. Results and discussion

The sulfonylurea drugs of interest in this study are generally considered to be weak acids with pK_a values in the 5.0–6.4 range [31,32] and, therefore, are expected to behave as non-polar, weakly charged analytes. Since CZE of these analytes was initially unsuccessful, an MEKC method has been developed [21] for their effective CE separation when extracted from urine containing the drugs at concentrations in the 100–500 ng/ml range (Fig. 1A). The goal of the present study was to extrapolate these separation conditions for use with the SPE–CE system that had been shown to be effective for the direct analysis of ng/ml levels of peptides [33]. It was thought that, with the SPE–CE approach, it may be possible to circumvent part of the labor-intensive off-line extraction procedure currently required for detection with the MEKC-based system. However, it was identified early in the study that the presence of surfactant (75 m*M* sodium cholate) in the separation buffer, a critical component for effecting the resolution of these drugs, interfered with the solid-phase extraction process. Attempts to identify a lower surfactant concentration that would not interfere with the solid-phase extraction process but still allow for resolution of the mixture components were nonproductive. Fig. 1B shows the ineffective separation that was obtained when the cholate concentration in the buffer was reduced.

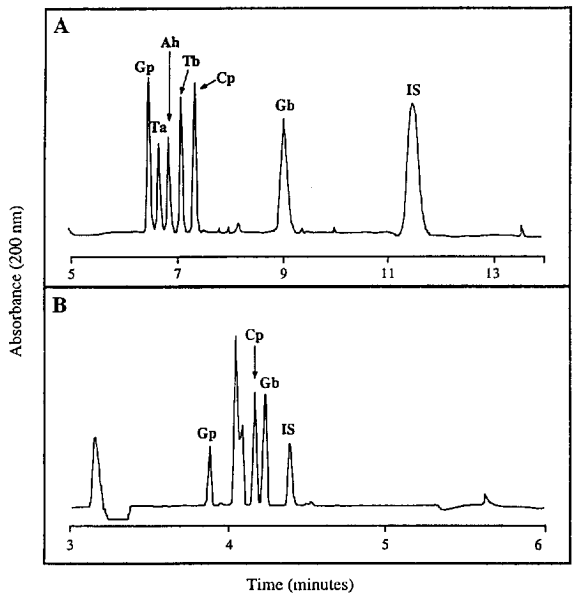

Fig. 1. MEKC separation of six sulfonylurea drugs and the internal standard. Separation was carried out in 5 m*M* borate–5 m*M* phosphate containing sodium cholate (25 kV) following a 2 s hydrodynamic injection. (A) Standard drugs (143 μg/ml), 75 m*M* cholate. (B) Standard drugs (143 μg/ml), 10 m*M* cholate. Separation was carried out in a 47 cm × 50 μm capillary thermostatted at 24°C at 25 kV.

Therefore, it was clear that surfactant-free conditions (i.e., non-MEKC) would be required.

Under the initial non-MEKC conditions tested, glipizide and glyburide could not be resolved. Glyburide has been shown to have a pK_a in the range 6.15–6.4 [34] while that for glipizide is 5.94 [35]. This is consistent with the observation that, at higher pH (8.50), Gb and Gp co-migrate slower than the EOF indicating that they are similarly charged and net negative. In theory, Gp and Gb should be separable at a pH between their pK_as where they would be differentially charged. Electrophoresing in 100 m*M* phosphate buffer varying from pH 6.3 to pH 6.0 showed that Gb and Gp were only separable at a pH of 6.0 (data not shown), at which point Gb co-migrates with the EOF (indicating that it had become uncharged). While these conditions were found to resolve Gb and Gp, the separation was extremely sensitive to slight changes in buffer pH. As a result, extensive electrophoresis (multiple runs in the same separation buffer) led to run-to-run reproducibility problems. Furthermore, having Gb co-migrate with the EOF was of limited use from the perspective of clinical assay development, since it would not be resolved from any of the uncharged (or neutral) compounds present in the sample. As a result, other conditions were sought for separation of Gb and Gp.

Through the testing of a number of buffer systems, it was eventually determined that Gp and Gb could be partially resolved at a pH higher than the pK_a range of these two drugs. Fig. 2A shows that 100 m*M* phosphate buffer, pH 7.07, containing 20% methanol allowed for the separation of Gp and Gb as well as five of the six other components in the mixture. Both the presence of methanol and the concentration of phosphate used in the buffer were found to be essential for this separation: use of a similar buffer containing 50 m*M* phosphate led to inadequate resolution. Unfortunately, Ta and I.S. were not resolved under these conditions and this was overcome by slightly altering the pH (in the presence of 20% methanol) (Fig. 2B). The analysis time (20 min) associated with separation of the mixture under these conditions was considered to be undesirable. The analysis time was decreased by shortening the effective length of the capillary from 40 cm to 20 cm which allowed for baseline resolution of Ta, I.S., Ah, Tb and Cp, and partial resolution of Gb and Gp (Fig. 2C). The accompanying analysis time of less than 6 min was competitive with that attainable using the MEKC-based analysis [21]. These conditions were considered to be adequate and were used in the initial attempts to implement SPE–CE for

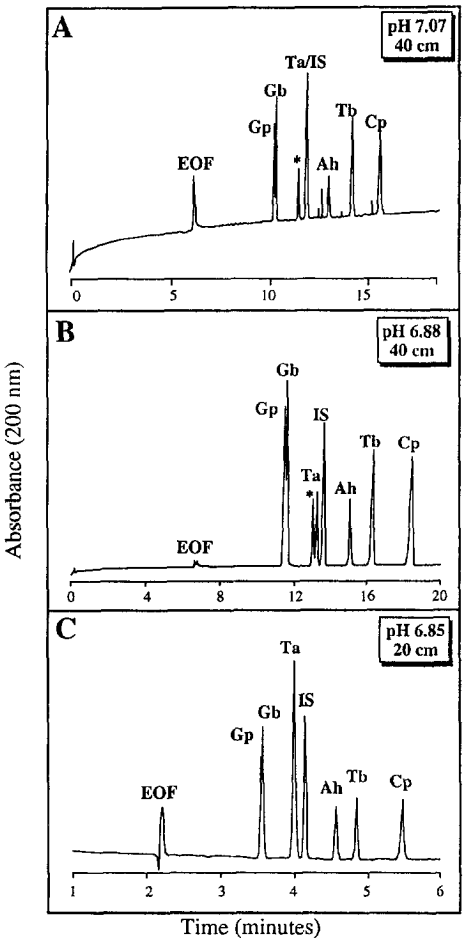

Fig. 2. CZE (non-MEKC) separation of sulfonylurea drugs and the internal standard. Separation was carried out in 100 m*M* phosphate–20% methanol at the designated pH (15 kV, 184 μA) following a 2 s hydrodynamic injection of the standard mixture (143 μg/ml) into a 47 cm × 50 μm (A and B) or 27 cm (C) capillary thermostatted at 24°C. Asterisk indicates a degradation product of Ah.

the concentration and separation of these analytes.

Solid-phase extraction–capillary electrophoresis (SPE–CE) was initiated with the construction of a SPE-tip from common laboratory materials as shown in Fig. 3. Initial attempts to extrapolate the non-MEKC conditions defined in Fig. 2 to SPE–CE were found to be only partially successful. Following injection of a mixture containing all seven compounds into the SPE-capillary, only Gb and Gp (which were unresolved) and I.S. appeared to be retained by the SPE-tip and desorbed with an elution buffer injection prior to electrophoresis (Fig. 4). It became clear that, when the SPE-tip and hybrid capillary were rinsed with the separation buffer containing 20% methanol prior to electrophoresis, Cp, Ta, Ah, and Tb were selectively desorbed from the solid phase and, therefore, were not detectable. It also became clear that the separation buffer should have two important characteristics: it should (1) not induce premature desorption of the analytes from the SPE-tip prior to elution buffer injection and (2) provide

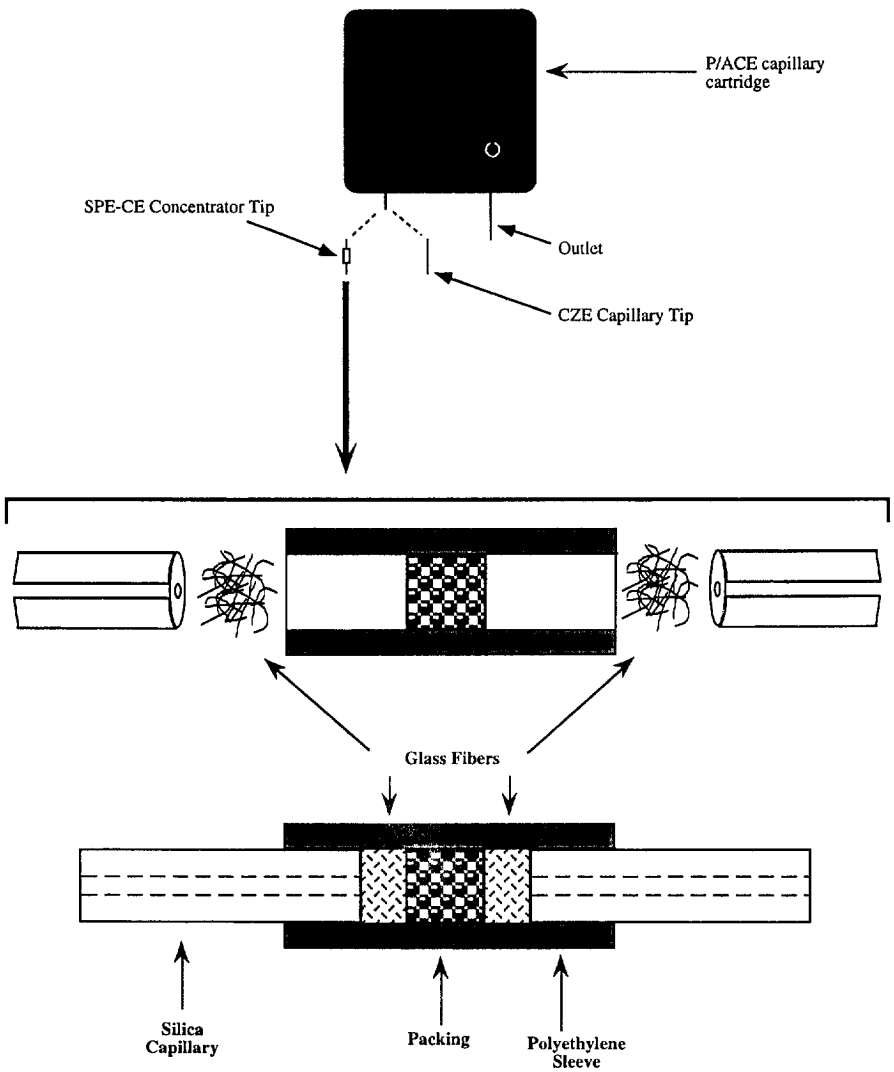

Fig. 3. Diagrammatic representation of the solid-phase extraction tip construction and placement.

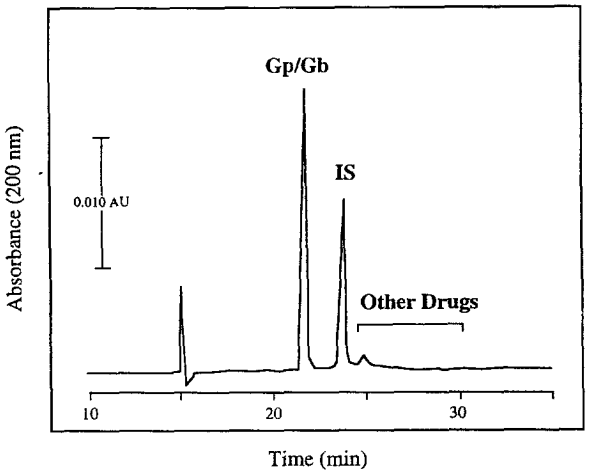

Fig. 4. Concentration and separation of hypoglycemic drugs on an SPE-tip containing a 0.5 mm × 370 μm C_{18} packing. A standard mixture was prepared containing the seven compounds at 143 μg/ml. The solution was loaded onto the SPE-tip by pressure for 0.2 min at $1.38 \cdot 10^5$ Pa. Elution buffer was 20% 50 m*M* phosphate, pH 2.5–80% ACN and was injected for 0.20 min at $3.45 \cdot 10^3$ Pa followed by a second injection of separation buffer for 0.5 min at $3.45 \cdot 10^5$ Pa. Separation was then carried out at 10 kV in 50 m*M* phosphate buffer–20% methanol pH 7.1 in a 47 cm × 75 μm capillary maintained at 20°C.

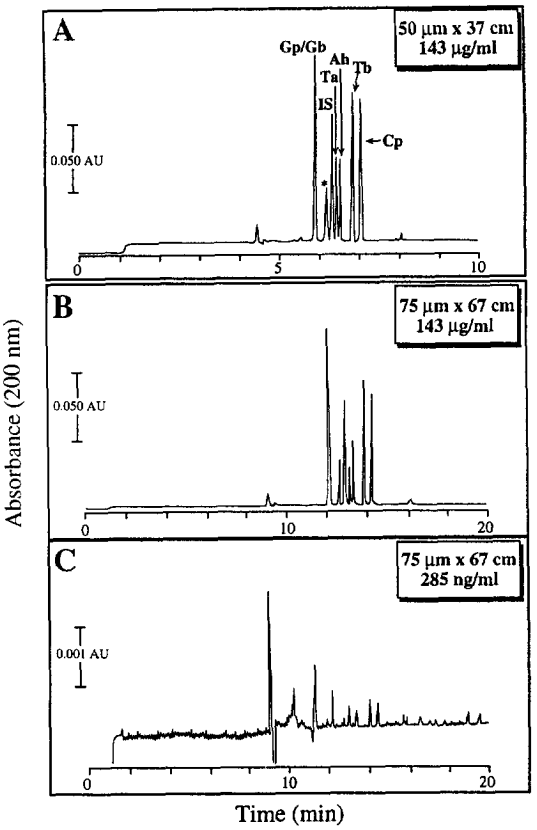

Fig. 5. Standard CZE of the sulfonylurea drugs in 250 m*M* borate–5 m*M* phosphate buffer, pH 8.4. A standard drug mixture was prepared by mixing equal volumes of each drug from 1 mg/ml stock solutions in methanol. This solution was diluted in water to the final concentrations shown in the figure. The solution was injected onto the capillary by pressure for 2 s at $3.45 \cdot 10^3$ Pa. Separation was then carried out at 20 kV while the capillary specified in the figure was maintained at 20°C.

adequate separation of the analytes of interest. After testing several buffer systems, an aqueous 250 m*M* borate–5 m*M* phosphate buffer, pH 8.4, was chosen despite the fact that Gp and Gb were not resolved. Fig. 5A shows a standard CZE separation of the drug mixture under these conditions in a 37 cm × 50 μm capillary. Since SPE–CE has been found to be more effective in longer capillaries with larger internal diameters [33], analysis of the same sample was carried out in a 67 cm × 75 μm capillary fitted with a CZE-tip (i.e., no SPE-tip) (Fig. 5B). These separations serve as reference for comparison between the CZE and SPE–CE methods and establish the integrity of the separation capillary without the SPE-tip attached. Seven baseline-resolved peaks were evident, with Gb and Gp co-migrating as a single peak and a degradation product of Ah providing the seventh response as indicated by the asterisk in the figures. When the sample was diluted 500-fold in water (final concentration of 285 ng/ml) and a replicate CZE analysis performed, the electropherogram presented in Fig. 5C was observed. At this dilution, the concentration of the drugs in the sample closely approximated the concentration that would be present in an unconcentrated urine specimen. This highlights the unsuitable nature of standard CZE for the direct analysis of the drugs without some form of off-line concentration. The 67 cm × 75 μm capillary was then fitted with the SPE-concentrator tip and the 500-fold diluted sample introduced into the inlet of the capillary in a large volume (≈2.5 μl by a timed

pressure injection). This represented a sample volume equal to the total inlet-to-detector volume (2.5 μl). The capillary and attached SPE-tip were then rinsed with several capillary volumes of the separation buffer while the drugs remained immobilized on the solid phase. Elution of the drug mixture from the solid phase was accomplished with a timed injection of elution buffer which varied in either composition or volume and electrophoresed (Fig. 6). As the injection time was decreased from 1 min to 0.5 min using 20% separation buffer–80% acetonitrile as elution buffer, the resolution increased markedly (Fig. 6A,B). Generally, the best separations (approaching the performance of standard CZE) were obtained by injecting the minimal volume of elution buffer (0.33 min; 60 nl) required to release the most hydrophobic of the analytes, i.e. Gb, Gp, and I.S. The elution volume was found to be critical to the efficiency of the separation since too little elution buffer resulted in selective retention of some of the analytes on the solid phase and excessive elution volumes precluded electrophoretic resolution of the analytes [36]. Inclusion of a low pH component in the elution buffer dramatically improved the peak shape and response (Fig. 6C). Comparing Fig. 5C to Fig. 6C highlights the power of direct extraction and concentration of the drug mixture on a solid phase resin that is in-line with the separation capillary.

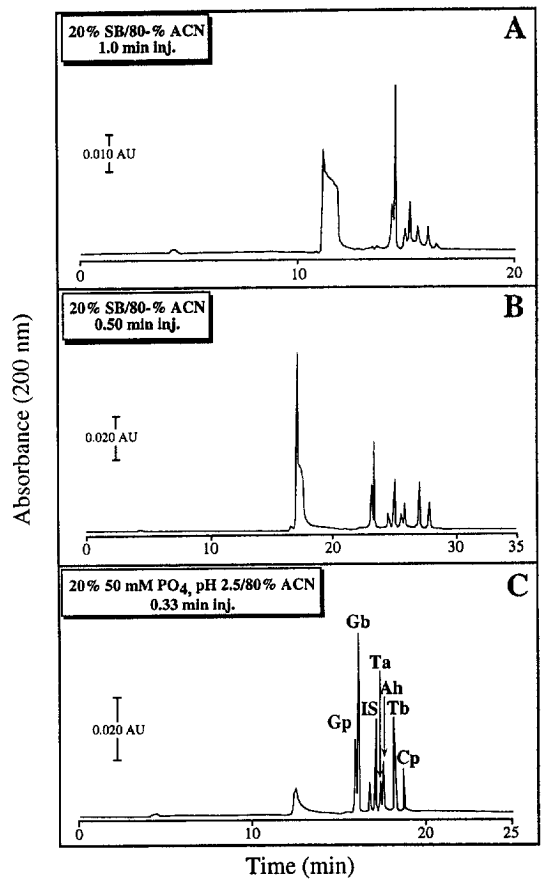

Fig. 6. The effect of elution buffer composition and volume on the desorption of analytes from the SPE tip. Sulfonylurea drugs (285 ng/ml in water) were loaded onto the SPE-tip for 0.2 min at $1.38 \cdot 10^5$ Pa and the packing washed for 1 min with separation buffer (SB; 250 mM borate–5 mM phosphate). The drugs were then desorbed by injection of an elution buffer as indicated in the figure. A $3.45 \cdot 10^3$ Pa injection of separation buffer preceded electrophoresis at 15 kV, 70 μA in a 67 cm × 75 μm capillary at 20°C.

The enhanced separation efficiency associated with the inclusion of a low pH buffer in the elution buffer is interesting. This may be due, in part, to the suppression of turbulence at the interface of the elution and separation buffers, a phenomenon previously reported by Chien and Burgi [37] that is believed to be caused by the mismatch of local EOF velocities in the discontinuous buffer system. In theory, this effect may cause a mixing and laminar backflow at the interface of the buffers and seriously degrade resolution and peak shape. Alternatively, pH stacking may be occurring due to the pH step gradient produced by the low pH elution buffer injection. Further study of this effect and its potential impact on SPE–CE separations is underway. It is important to note that enhanced separation of Gp and Gb was also accomplished without the addition of MeOH to the separation buffer. This may provide evidence that, despite the small bed volume in the SPE-tip (≈50 nl), some chromatography occurs during the desorption of the drugs from the solid phase. These chromatographic effects can have positive effects or negative effects [33] on the separation. In this

particular case, the chromatographic component appears to have been beneficial to the separation.

Reproducibility and SPE-tip lifetime for this particular application was determined using a fresh SPE-capillary containing a 0.5 mm × 370 μm packed bed of C_{18} reversed phase and a 47 cm × 75 μm (40 cm to detector) separation capillary. The flow-rate of the capillary was calculated by injection of an aqueous buffer at the inlet of the capillary (using $1.38 \cdot 10^5$ Pa) and measuring the time required for the buffer to reach the detector. The following was observed in this particular case: time to detector, 0.19 min at $1.38 \cdot 10^5$ Pa; inlet to detector volume, 1.77 μl plus an estimated void volume of ≈50 nl in the concentrator tip; calculated flow-rate, 9.56 μl/min. This estimated flow-rate correlated well with the observed rate of sample consumption and was used as a general indicator of the physical integrity of the SPE–CE capillary. The flow-rate of the capillary was found to decrease slightly (≈10% based on rinse buffer inlet to detector times) during the first few separations and then remained stable. These separations were considered to be a "break-in" period for the SPE-tip. To shorten the analysis time, the separation buffer concentration was decreased to 125 m*M* borate/2.5 m*M* phosphate, pH 8.4, which still provided adequate resolution for the two-drug mixture. The SPE-tip performed well for a minimum of 30 consecutive analyses with regeneration of the reversed phase being accomplished with a short (0.5 min at $1.38 \cdot 10^5$ Pa) rinse using 80% ACN–20% buffer. At the completion of this set of experiments, the SPE-tip was intentionally-overloaded with sample – 5 min at $1.38 \cdot 10^5$ Pa with 2 μg/ml; total 48 μl or 96 ng – which prematurely destroyed functionality. In other experiments, an SPE-tip was found to easily perform up to 50–100 consecutive analyses under conditions where sample overloading was avoided. Run-to-run migration time was found to be relatively reproducible although minor shifts in the migration time became evident over several consecutive separations. This shift is negligible from run to run but obvious when comparing analyses separated by several runs. This is illustrated in Fig. 7 which shows the sixth and fifteenth separation of the same sample, and the slight shift in migration time associated with consecutive use. The shift is likely the result of the inability to rinse the capillary with 0.1 *M* NaOH which is avoided to prevent destroying the solid phase.

To determine the sensitivity (detection limit) associated with this particular application of the SPE–CE technology, a simple two-component mixture containing Gp and I.S. was employed to (1) ensure that, at low levels, resolution was

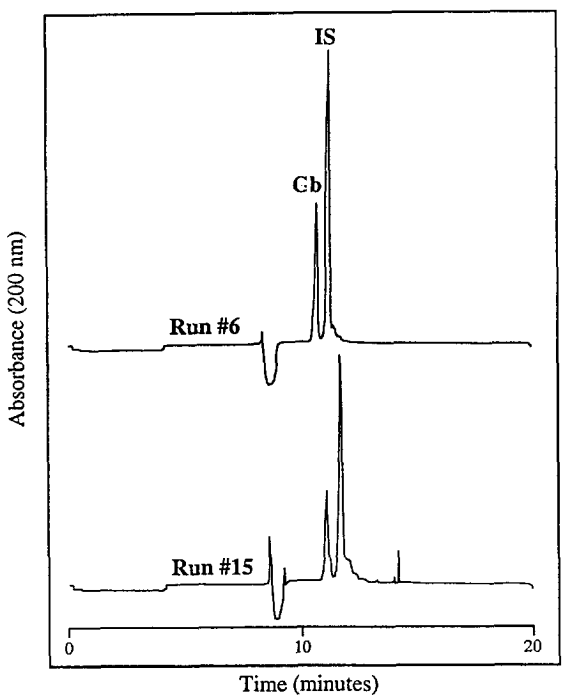

Fig. 7. Reproducibility associated with consecutive use of an SPE tip. Concentration and separation of sulfonylurea drug Gp and I.S. at 2 μg/ml on an SPE capillary containing a 0.5 mm × 370 μm C_{18} packing. Sample buffer solution was loaded onto the SPE tip by low pressure $3.45 \cdot 10^3$ Pa, followed by a separation buffer rinse. The separation buffer was 20% 50 m*M* phosphate (pH 2.5)–80% ACN and was injected for 0.30 min at $3.45 \cdot 10^3$ Pa followed by a second injection of separation buffer for 0.5 min at $3.45 \cdot 10^3$ Pa. Separations were then carried out at 15 kV in 125 m*M* borate–2.5 m*M* phosphate pH 8.0 buffer in a 47 cm × 75 μm capillary maintained at 28°C.

unaffected in comparison with the standard CZE electrophoretic separation and (2) determine whether samples serially diluted 10-fold (2 μg/ml, 200 ng/ml and 20 ng/ml) could be applied directly onto the SPE concentrator. The data in Fig. 8 demonstrates that detection of both Gp and I.S. as low as 20 ng/ml was easily attainable, but that extraction efficiency drops dramatically (apparently exponentially) with dilution beyond this point. It is noteworthy that the extraction efficiency is markedly different for Gp and I.S. and that further dilution of the sample resulted in complete loss of I.S. detectability. This is not surprising since differences in the inherent affinities of analytes for reversed phases is the basis for chromatographic separations. Provided that resolution is not affected, increasing the packed-bed volume should increase the sensitivity of the technique for the Gb as demonstrated in Fig. 9 where 190 μl of sample has been concentrated and detected. Note that the data in this figure was generated using the same 47 cm × 75 μm separation capillary utilized in the other experiments but with an SPE-tip that had a reversed-phase volume that was four times that used in the experiments evaluating lifetime and

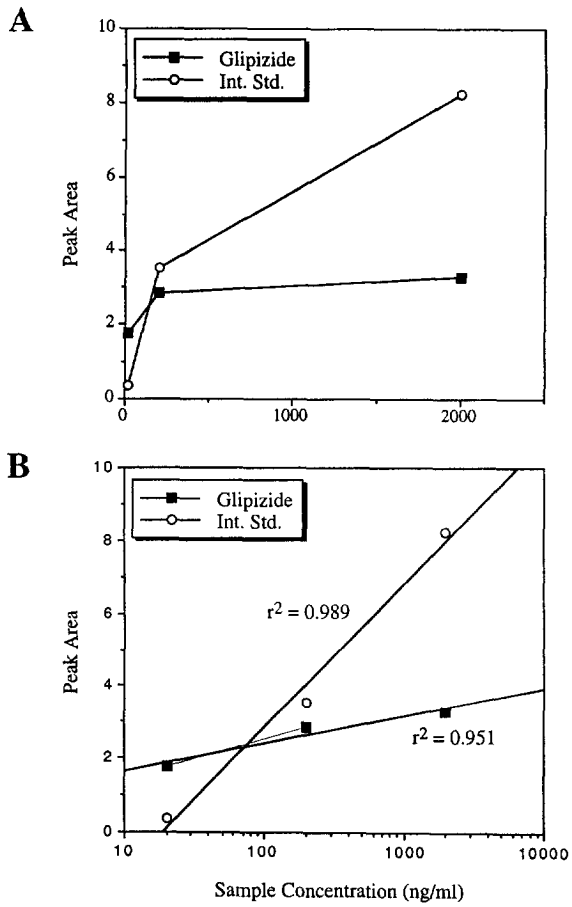

Fig. 8. Extraction efficiency vs. sample concentration. Concentration and separation of Gp and I.S. after sample loading of 2.0, 0.2 and 0.02 μg/ml at 0.05, 0.5 and 5.0 min injection times on an SPE capillary containing a 0.5 mm × 370 μm C_{18} packing. Sample buffer solution was loaded onto the SPE tip by low pressure ($3.45 \cdot 10^3$ Pa) followed by a separation buffer rinse. The elution buffer was 20% 50 m*M* phosphate, pH 2.5–80% ACN and was injected for 0.30 min at $3.45 \cdot 10^3$ Pa followed by a second injection of separation buffer for 0.5 min at $3.45 \cdot 10^3$ Pa. Separations were then carried out at 15 kV in 125 m*M* borate–2.5 m*M* phosphate pH 8.0 buffer in a 47 cm × 75 μm capillary maintained at 28°C.

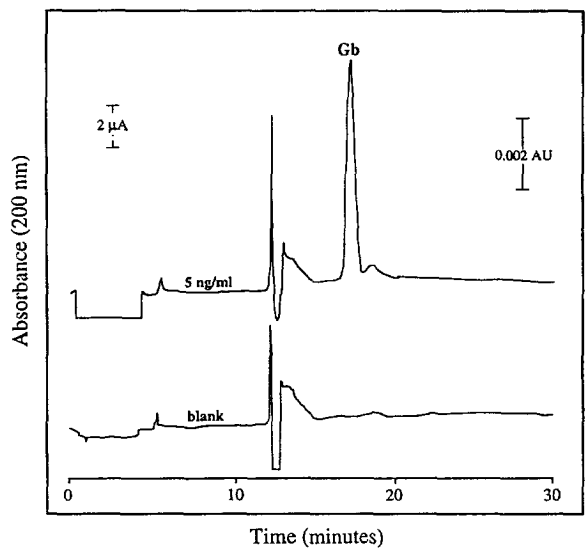

Fig. 9. Detection of Gb at 5.0 ng/ml using SPE–CE. Concentration and separation of sample blank and Gb on an SPE capillary containing a 1.0 mm × 370 μm C_{18} packing. Sample was loaded onto the SPE tip by high pressure ($1.38 \cdot 10^5$ Pa) for 20 min (equivalent to injection of 190 μl) followed by a separation buffer rinse. Elution buffer was 20% 50 m*M* phosphate, pH 2.5–80% ACN and was injected for 0.30 min at $3.45 \cdot 10^3$ Pa followed by a second injection of separation buffer for 0.5 min at $3.45 \cdot 10^3$ Pa. Separation was then carried out at 15 kV in 125 m*M* borate–2.5 m*M* phosphate pH 8.0 buffer in a 47 cm × 75 μm capillary maintained at 28°C.

loading capacity. The larger volume of packing allowed for an increase in sensitivity but, if excessive, resolution may be lost as a result of the increased elution volume required to release the bound analytes.

The sample loading capacity was determined using a single concentration of Gp and I.S. (2 μg/ml) and by doubling the injection time over a range of 0.5–32.0 min using an applied injection pressure of $3.4 \cdot 10^3$ Pa. The peak area was found to increase linearly with injection time over the range of 0.5–8.0 min with a calculated correlation coefficient (r^2) of 0.999 for both Gp and I.S. (Fig. 10). With injections of 16.0 and 32.0 min, the resultant recovery of Gp and I.S. was no longer linear, presumably due to the fact that the capacity of the solid phase was approached ($\approx$15 ng of each drug loaded with an injection of 32 min at $3.4 \cdot 10^3$ Pa). When an extremely large volume of sample was injected (5.0 min at $1.38 \cdot 10^5$ Pa = 47.8 μl), the capacity of the concentrator tip was exceeded and the exchange of I.S. for Gp on the solid phase was observed as evidenced by the corresponding decrease in I.S. peak area as the peak area of Gp continued to increase.

As a means of evaluating other phases with different physical characteristics, an SPE-tip was constructed with a Poros R2 resin provided by PerSeptive Biosystems. This solid-phase packing was unique in that it is extremely stable over a wide pH range (pH 1–14). Concentration and separation on a Poros R2 SPE-tip showed that, while the binding affinity of the drugs to this resin was apparently lower than that with the C_{18} silica, the SPE-tip was not affected by the use of high pH buffers or conventional reconditioning techniques (i.e., 0.1 *M* NaOH buffers) that typically necessitated the renewal of the silica-based SPE tips (data not shown). The Poros material also allows for high flow-rates and low restriction due to the favorable physical characteristics of this support, and thus, is a favorable solid phase for use in SPE–CE methods. Unfortunately, the additional chromatographic component observed with the silica-based packings could not be reproduced with the Poros solid phase. This may be an indication of the different selectivities that can be expected with different packings. While this solid phase may not be perfectly suited to this particular application (these analytes), it may be better suited to other more hydrophobic analytes.

4. Conclusions

The results of this study indicate that SPE–CE appears to be effective for the analysis of the hypoglycemic drugs in the low ng/ml range. The ability to inject and concentrate up to 190 μl of sample on a system that is typically restricted to a maximum injection of 25 nl, translates to an increase in sensitivity of approximately 3–4 orders of magnitude. This sensitivity should, in theory, allow for the development of a method for the direct detection of hypoglycemic drugs in urine at 100–500 ng/ml. Sample loading was linear until the capacity of the solid support is approached and the SPE-tip was shown to be robust in that it is capable of multiple consecutive analyses with minimal regeneration. The ability to automate the sample, elution and buffer injections as well as the electrophoretic process are critical for effective application of this technology to clinical assay development. The potential for elimination of off-line extraction procedures presently required prior to analysis by CE or other standard techniques is enticing. Solid supports with different adsorption/desorption selectivities may be utilized to produce customized methods for individual analyses. Therefore, the use of a diverse array of solid-phase packing materials will impart a tremendous flexibility and range to this CE technique.

Acknowledgments

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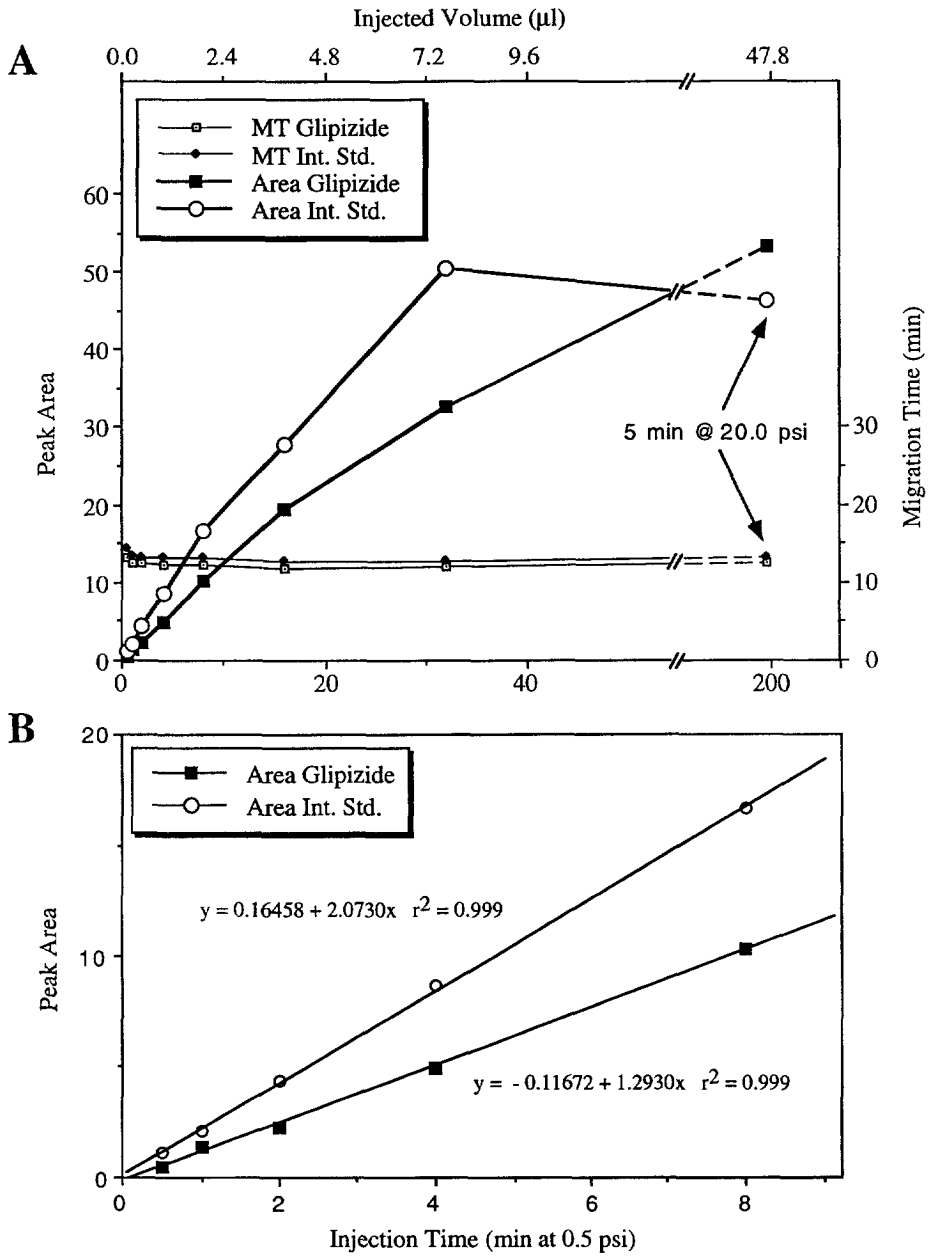

Fig. 10. Extraction efficiency vs. injection time. (A) Linearity of extraction efficiency vs. injection time: Concentration and separation of sulfonylurea drug Gp and I.S. at 2 μg/ml on an SPE capillary containing a 0.5 mm × 370 μm C_{18} packing. Sample buffer solution was loaded onto the SPE tip by low pressure ($3.45 \cdot 10^3$ Pa), except where noted in figure, followed by a separation buffer rinse. Elution buffer was 20% 50 m*M* separation buffer–80% ACN and was injected for 0.30 min at $3.45 \cdot 10^3$ Pa followed by a second injection of separation buffer for 0.5 min at $3.45 \cdot 10^3$ Pa. Separations were then carried out at 15 kV in 125 m*M* borate–2.5 m*M* phosphate pH 8 buffer in a 47 cm × 75 μm capillary maintained at 28°C. (B) Correlation of peak area vs. injection time with injections over a range of 0.5–8.0 min.

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