Background: The diagnosis of “factitious hypoglycemia” is essentially based on the disclosure of hypoglycemic agents in blood or urine. The aim of this study was to evaluate the performance of capillary electrophoresis (CE) as a quantitative method for determination of chlorpropamide, tolbutamide, glipizide, gliclazide, and glibenclamide in serum.

Methods: Serum samples (1 mL), with internal standard added, were purified by solid-phase extraction on OASIS™ HLB cartridges (Waters), dried under reduced pressure, and reconstituted with 30–60 µL of acetoni-trile:H2O. Analysis was carried out by micellar electrokinetic capillary chromatography in 5 mmol/L borate, 5 mmol/L phosphate, 75 mmol/L sodium cholate, pH 8.5, containing 25 mL/L methanol. Separation was accomplished in a 20 cm × 50 µm (i.d.) silica capillary at 25 °C and a constant voltage of +10 kV. Pharmacokinetics of gliclazide (80-mg tablet) in a diabetic patient were assayed by both HPLC and CE. Two hypoglycemic patients positive by HPLC analysis for unreported gliclazide and tolbutamide overdose were also screened by CE.

Results: Separation of six drugs (including the internal standard) was accomplished in 5 min plus 5 min rinsing. The between-day CV of the ratio of the areas of the sulfonylurea drugs to internal standard was <1% (n = 10). Linearity (r² ≥ 0.998) and recovery (≥80%) were good for all sulfonylurea drugs tested. Pharmacokinetic curves for gliclazide by CE and HPLC were superimposable. CE analysis confirmed the HPLC diagnosis of surreptitious abuse of gliclazide and tolbutamide.

Conclusion: CE is a useful tool in the clinical chemistry and toxicology laboratory for drug monitoring and pharmacokinetic investigations.

Symptomatic fasting hypoglycemia is a severe and potentially life-threatening condition usually triggered in otherwise healthy adults by exaggerated fasting insulin concentrations (1). The most common causes of hyperinsulinism include insulin-secreting pancreatic β-cell tumors, surreptitious administration of sulfonylurea (SU)5 drugs or insulin, overdose of antidiabetic medication, autoimmune disorders, and insulin reaction (in diabetic patients) (2–5). The diagnosis of insulin-secreting tumors relies mainly on a biochemical profile: because of their often small size (<2 cm), localization by ultrasonography, computed tomography scanning, or magnetic resonance imaging may be misleading (6, 7). Serum insulin ≥10 mIU/L (10 µIU/mL) associated with plasma glucose values <2.5 mmol/L (45 mg/dL) suggests the presence of insulinoma. However, surreptitious administration of insulin or SU drugs cannot be ruled out, especially in health professionals or relatives of a diabetic patient (8). “Factitious hypoglycemia” is confirmed when the origin of the insulin increase is demonstrated to be exogenous (suppressed plasma C-peptide immunoreactivity) or when SU drugs (or their metabolites) are found in serum or urine (9-12). Timely discovery of an unre-
ported overdose of SU drugs could avoid unnecessary exploratory surgery or a partial pancreatectomy.

Because of the importance of accurately diagnosing self-administration abuse, we routinely use HPLC for identification of SU drugs in the blood of suspected patients. The procedure is based on previously published methods (13–15) and has been optimized for identification, in a single analysis, of the most commonly prescribed SU drugs, including tolbutamide (TL), chlorpropamide (CL), glipizide (GP), gliclazide (GL), and glibenclamide (GB). Positive samples are always re-analyzed by an HPLC method based on pre-column derivatization with fluorodinitrobenzene (16, 17). When some doubt remains, confirmation by mass spectrometry is mandatory.

Capillary electrophoresis (CE) is an analytical technique whose popularity is quickly increasing in the clinical chemistry laboratory as an alternative to traditional gel electrophoresis for proteins, peptides, and oligonucleotides. In particular, a micellar electrokinetic capillary chromatography (MEKC) method, based on the use of surfactant agents, has been specifically designed for the separation of neutral and uncharged analytes (18, 19), and its use as an alternative method for identification of SU drugs in urine has been described (20, 21). None of the above-mentioned studies, however, explored the performance of MEKC for determination of SU drugs in serum, and none reported in detail a quantitative evaluation.

The aim of this study was to evaluate the potential of MEKC both as a qualitative assay to help in identification of SU drugs in serum for rapid and accurate diagnosis of drug-induced hypoglycemia and as a quantitative technique to be used in pharmacokinetic investigations. For this purpose, we assessed serum pharmacokinetics of GL (80-mg tablet) in a type 2 diabetic patient by both HPLC and CE. Moreover, two patients investigated for factitious administration abuse, we routinely use HPLC for identification of SU drugs in the blood of suspected patients.

Materials and Methods

MATERIALS

Sodium borate, boric acid, sodium hydroxide, monosodium dihydrogen phosphate, and disodium hydrogen phosphate were from Sigma Chemicals. CL, TL, GB, and GP were from ICN Pharmaceuticals. GL was a gift from Molteni Farmaceutici, Florence, Italy. N-Acetyl-5-(2,3-dichlorophenylureido)benzenesulfonamide was purchased from Aldrich Chemical. HPLC-grade methanol, acetonitrile, and toluene were from BDH Laboratory Supplies. Sodium cholate was purchased from Fluka. OASIS™ HLB extraction cartridges (3 mL, 60 mg) were from Waters. The fused-silica capillary was from Beckman Instruments.

PREPARATION OF BUFFERS AND CALIBRATORS

The buffer for HPLC separation was 10 mmol/L potassium hydrogen phosphate, pH 3.5. Stock borate buffer (0.5 mol/L), pH 8.5, and phosphate buffer (0.5 mol/L) for CE analysis were prepared as described previously (20, 21). We prepared CE running buffer by diluting the stock buffers 1:100 (by volume) and adding 75 mmol/L sodium cholate and 25 mL/L methanol. The CE washing buffer was prepared in the same way except that the stock buffer was diluted 1:10.

Stock drug solutions were prepared at 1 g/L in methanol and kept at −20 °C. Working solutions containing 0.2, 0.05, 0.02 g/L of each drug were prepared by dilution in doubly distilled water.

LIQUID-LIQUID SERUM EXTRACTION

Serum samples (1 mL) were added to 10 μL of the internal standard solution (see below) and acidified with 0.2 mL of 1 mol/L HCl. Extraction was accomplished by mixing with toluene (5 mL) for 15 min. After centrifugation for 5 min at 3000 g, the organic phase was transferred into a conical screw-capped glass tube and dried in a vacuum centrifuge (Savant).

SOLID-PHASE SERUM EXTRACTION

Serum samples (1 mL) were acidified with 0.2 mL of 1 mol/L HCl, added to 10 μL of the internal standard solution (see below), and diluted 1:1 (by volume) with water. The OASIS HLB extraction cartridges were connected to Visiprep Solid Phase Extraction Vacuum Manifolds (Supelco), and after pressure was reduced with a water pump, the cartridges were preactivated by washing with 1 mL of methanol followed by 1 mL of water (~1 mL/min). The diluted serum samples were loaded onto the columns and then washed with water (1 mL), followed by a series of water:methanol mixtures [1 mL of 95:5 (by volume), 1 mL of 80:20, 1 mL of 70:30, and 1 mL of 60:40]. After air was flushed through the columns for 1 min, the drugs of interest were eluted with 1 mL of methanol:acetonitrile (1:1, by volume) and dried under reduced pressure.

HPLC ANALYSIS

A Kontron instrument composed of two pumps (model 420), an autosampler (model 460), and a double-beam ultraviolet detector (model 430) set at 225 nm was used. Kroma System 2000 software was used for data handling. The column was a LiChrospher 100 RP-18 [25 cm × 4.5 mm (i.d.); 5 μm bead size; Merck]. The dried samples were reconstituted with 120 μL of K2HPO4:methanol (40:60, by volume), and 20–100 μL was injected into the column. A good compromise between rapidity of analysis and resolution of TL, CL, GP, and GL was achieved simply by eluting the column with 600 mL/L methanol in potassium phosphate buffer. To detect the presence of GB, however, a gradient to 710 mL/L methanol was necessary (see Fig. 1). To further improve the separation of the first eluting peaks, the analysis was sometimes started at 580 mL/L methanol.
CE separation was carried out using a P/ACE 5010 System (Beckman Instruments) equipped with a monochromatic ultraviolet detector set at 200 nm and controlled by System Gold 8.1 software. The fused-silica capillary [27 cm × 50 μm (i.d.); 20 cm to the detector] was assembled in a Beckman cartridge (200 × 400 μm slit aperture). For a typical analysis, the following procedure was used: 3-min prerinse with the running buffer (20 psi, 138 kPa), 2- to 5-s injection of the sample at low pressure (0.5 psi, 3.4 kPa), 1-s pressure injection of running buffer, separation at +10 kV (37 μA), 1-min rinse with the washing buffer, followed by 1 min with 0.1 mol/L NaOH. The capillary temperature was maintained at 25 °C. The dried samples were reconstituted with 30–60 μL of acetone:trifluoroacetic acid (60:40, by volume) and loaded into the water-cooled autosampler tray.

**Calibration Curves for CE Analysis**

Calibration curves were prepared with the working solutions. We prepared a GL calibration curve in the 1–20 mg/L range by adding to 1 mL of control serum increasing amounts of GL (5–100 μL of a 0.2 g/L solution), and a fixed amount of TL used as internal standard (10 μL of a 0.2 g/L solution; total amount added, 2 μg). The samples were then acidified and loaded on the solid-phase extraction cartridge as described previously. We prepared a calibration curve for GB in the 0.25–20 mg/L range using the 0.05 and 0.2 g/L solutions. For TL and CL determination, N-acetyl-5-(2,3-dichlorophenylureido)benzenesulfonamide was added as internal standard (10 μL of a 0.5 g/L solution; total amount added, 5 μg). The curves were in the 0.4–20 mg/L range, and the 0.02 and 0.2 g/L solutions were used (20–100 μL). Because of the small volumes of patients’ sera available, some curves were prepared as described, but only 0.2 mL of plasma was extracted and analyzed.

**Pharmacokinetics of GL in Serum**

To determine whether CE could give reliable results in identification and quantification of SU drugs, blood from a type 2 male diabetic patient under chronic treatment with GL (40 mg three times per day before meals for 1 year) was withdrawn immediately before oral intake of a 80-mg tablet in the morning, and after 0.5, 1, 2, 3, 4, 6, and 8 h. During the study the patient had regular meals.

**Patients**

We studied two cases of hypoglycemia after overdose of factitiously administered SU drugs.

Patient 1 was a 58-year-old woman who attended the outpatient clinic because of severe obesity. She complained of tachycardia, sweating, weakness, and recent hypoglycemic episodes. An insulin-secreting tumor was suspected, but after she was hospitalized for 6 days, the cause of the hypoglycemia had not been diagnosed. The patient’s husband had diabetes and used GL. A screen to confirm surreptitious abuse of SU drugs was requested.

Patient 2 was a 44-year-old woman affected by posttraumatic epilepsy, asthma, chronic gastritis, and depression. She was hospitalized for evaluation of recent hypoglycemic episodes. Her chronic medications included phenobarbital, ranitidine, diazepam, salmeterol xinafoate, and antacid. The patient had familial and psychological problems, and the father was a diabetic being treated with SU drugs. The search for an insulinoma gave negative results, and because of the clinical characteristics of the patient, covert use of SU drugs was strongly suspected. A serum sample was collected and sent for laboratory evaluation.

**Blood Samples**

Blood samples were collected in Vacutainer® Tubes without additives; after separation, the sera were stored at −20 °C until analysis.

**Results**

**Assay Validation**

The method used routinely in our laboratory for the diagnosis of abuse of SU drugs was based on liquid-liquid extraction with organic solvents (toluene), followed by HPLC analysis, as described previously by several authors (13–17). To introduce the CE method as a confirmatory method for SU identification in the cases of factitious hypoglycemia, we tried to optimize the preanalytical phase by replacing the classical liquid-liquid extraction with a solid-phase extraction (22). The best results were obtained with a novel sorbent, based on the “universal” OASIS HLB copolymer, designed to separate, in a single chromatographic run, a wide range of compounds with a greater capacity than the classical C18-bonded phase. The recoveries obtained for the different SU drugs in a control serum at 0.5, 2.0, and 10 mg/L are shown in Table 1. Because of the improved performance, all analyses reported below (by HPLC or CE) refer to the solid-phase extraction procedure.

A typical profile obtained by our reversed-phase HPLC method for screening of SU drugs is shown in Fig. 1. The

<table>
<thead>
<tr>
<th>Table 1. Recovery of SU drugs from serum by liquid-liquid and solid-phase extraction.*</th>
<th>Extraction recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>SU drug</td>
<td>Liquid-liquid</td>
</tr>
<tr>
<td>GL</td>
<td>60 ± 18</td>
</tr>
<tr>
<td>GB</td>
<td>58 ± 17</td>
</tr>
<tr>
<td>GP</td>
<td>61 ± 12</td>
</tr>
<tr>
<td>TL</td>
<td>65 ± 11</td>
</tr>
<tr>
<td>CL</td>
<td>66 ± 12</td>
</tr>
</tbody>
</table>

* Recovery was estimated by HPLC vs absorbance of pure SU calibrators. Values reported are the means of the recoveries obtained by adding, in triplicate, 0.5, 2, and 10 μg of each SU drug to 1 mL of serum and processing with the two procedures as described in Materials and Methods.
analysis time was 35 min, but good resolution of all drugs, including the strongly retained GB, was achieved. Fig. 2 shows the CE electropherogram of a mixture of five SU drugs plus N-acetyl-5-(2,3-dichlorophenylureido)benzensulfonamide, which has been suggested by Roche et al. (21) as a possible internal standard for analysis of urine by CE. It is not surprising that the elution order of the SU drugs obtained by CE is different from that obtained by HPLC: the resolution in the former technique is based on the distribution coefficient between the micellar and aqueous phases (the greater the percentage of the analyte that is distributed into the micelle, the slower it migrates). In both CE and HPLC, however, GB was the last compound to elute, likely displaying a higher affinity for C18-RP resin and higher capacity factor for the cholate micelle. When the MEKC conditions (slightly modified) described by Roche et al. (20) were used, the serum components sometimes interfered with the N-acetyl-5-(2,3-dichlorophenylureido)benzensulfonamide. We therefore preferred to use TL as internal standard for quantification of GL, GB, and GP. Peak identification was carried out in relation to the migration time (tM) for the internal standard and by comparison with an external calibrator, with the area corrected for the tM always used. Linearity by CE was good for all of the SU drugs. The regression equations were calculated by plotting the SU drug:internal standard area ratio vs the serum concentration, and the resulting correlation coefficients were all ≥0.998. Examples of the curves for GL, TL, and GB are reported in Fig. 3.

Under the described analytical conditions, blank serum
showed no interference for any of the SU drugs except for GB, for which there was always a peak present at the same $t_M$ corresponding to a concentration of $\sim 0.060$ mg/L, that had to be taken into account for correct quantification. The minimum detectable concentrations in serum were 0.2 mg/L for GB and 0.1 mg/L (signal-to-noise ratio, 3:1) for all other SU drugs. This value could be improved only minimally by increasing the injected amount because peak broadening together with comigration of interfering serum peaks occurred. The HPLC detection limit was much lower (5 $\mu$g/L) because it was possible to inject the entire extracted sample.

The reproducibility of CE system was determined by analyzing mixtures of three different SU drugs (0.066 g/L) 10 times a day and for 10 consecutive days. The relative standard deviation for the peak area and the $t_M$ was 2–5% for within-day and 5–7% for between-day reproducibility. When the SU areas were corrected for the internal standard [TL or N-acetyl-5-(2,3-dichlorophenylureido)benzenesulfonamide] the between-day CV for the area ratio dropped to $<1\%$ for all SU drugs tested. For quantitative purposes, the use of an internal standard is, therefore, essential.

**PHARMACOKINETICS OF GL IN SERUM**

The pharmacokinetic curves for GL in serum, assayed by HPLC and CE, are shown in Fig. 4. Despite some slight differences (1.0 vs 0.87 mg/L and 1.06 vs 0.93 mg/L for CE vs HPLC at 2 and 8 h, respectively), the two methods produced kinetic curves with the same trend and with the majority of sampling points almost superimposable, thus supporting the reliability of CE as a quantitative technique. The GL concentration in serum of the diabetic patient under examination, who had good metabolic control, was $0.7–1.2$ mg/L (23, 24).

**IDENTIFICATION OF GL-INDUCED HYPOGLYCEMIA**

The chromatographic profile obtained from the solid-phase-extracted serum (0.2 mL) of patient 1 is shown in Fig. 5. An important peak, not present in the control serum (data not shown) and corresponding to the retention time ($t_r$) of GL was evidenced ($\sim 11.8$ min). Quantification against the GL calibration curve in serum gave a concentration of 12.1 mg/L. The presence of GL in the serum of the patient was also confirmed by HPLC analysis with precolumn derivatization (data not shown). The same serum from the patient (0.2 mL) was reextracted and analyzed by CE. The electropherogram confirmed the presence of a peak that corresponded to the $t_M$ of GL calibrator (3.29 min) and was quantified as 12.6 mg/L (Fig. 6).

The patient repeatedly denied using SU drugs or any other medication, and we were not able to contact her any more after hospitalization.

**IDENTIFICATION OF TL-INDUCED HYPOGLYCEMIA**

The chromatogram obtained for the serum sample collected from patient 2 is shown in Fig. 7. The important peak at $t_r \sim 7.2$ min suggested covert self-administration of TL and was estimated to have a concentration of 11.4 mg/L. A small but significant peak was also detected at the $t_M$ of GB, and corresponded to a concentrations of 0.035 mg/L. The presence of TL and GB in the serum of the patient was also confirmed by HPLC with precolumn derivatization (data not shown). The CE profile from the
same solid-phase-extracted serum (Fig. 8) confirmed the presence of TL ($t_M = 3.55$ min) at a concentration 14.1 mg/L. The peak at $t_M = 3.77$ min, likely a concomitant therapeutic drug or a more polar metabolite, did not correspond to any of the SU drugs under evaluation. In the present setting, the CE technique did not have enough sensitivity to detect the presence of GB.

During the period when patient 2 was still hospitalized, some GB pills disappeared from the ward. On this occasion, a second blood specimen was withdrawn, and the presence of GB was also confirmed by mass spectrometric analysis. The patient was questioned but denied antidiabetic medication use. After 3 months of psychiatric therapy and social counseling, this patient’s hypoglycemic episodes disappeared and SU drug tests were negative.

**Discussion**

In this report, we present the potential of CE as a quantitative technique for the determination of SU drugs of the second (TL and CL) and the third (GB, GP, and CL) generation in serum and as a clinical assay for confirming surreptitious abuse. Using the MEKC method described by Roche et al. (21) for urine analysis, we achieved satisfactory resolution of six SU drugs with a shorter and less-expensive fused-silica capillary (20 cm vs 40 cm; uncoated vs coated). Moreover, although these authors did a very elegant and extensive study on qualitative identification of SU drugs and GB metabolites in urine, using the diode array detector, they reported only profiles from diabetic patients under drug therapy without check-
ing the potential of CE in discovering factitious intake of these drugs. Here we describe application of the CE technique to the quantification of SU parent drugs in serum, providing data on linearity, reproducibility, sensitivity, and a comparison with results by HPLC.

The CE technique seemed to offer several advantages compared with HPLC or mass spectrometry: it is very rapid (analysis time <6 min), relatively robust, inexpensive (low volumes of buffers and reagents), and quite reproducible with the use of a suitable internal standard. Coupling with a diode array detector or with a mass spectrometer may improve the accuracy of the assay. If these instruments are not easily accessible, we suggest use of CE as a secondary test for confirming HPLC-positive results.

When a suitable internal standard was used, CE was shown to be valid as a quantitative technique, displaying an excellent linear response for all of the drugs tested in the therapeutic and above-therapeutic range. CE also provided satisfactory performance when compared with HPLC, although because only very small volumes of patients’ sera were available, this evaluation was based on only one aliquot per sample extracted and analyzed with both methods. In the present setting, the only true limitation of this method appears to be the detection limit, which is too high for pharmacokinetic studies on the third generation of SU drugs, which because of their greater potencies are prescribed at very low dosages. Moreover, this could yield false-negative results if patients are investigated more than 24 h after ingestion, when SU drugs have been mostly metabolized and excreted in urine.

Kunkel and Wätzing (19), using direct injection of plasma samples for drug quantification by MEKC, found a dramatic improvement of the detection limit (to 0.005–0.010 mg/L) for acetaminophen and salicylate when they added 75–100 mL/L isopropanol to both the running and washing buffers. Despite the fact that we had already modified the buffer used by Roche et al. (21) by adding 25 mL/L methanol and that we performed preanalytical purification and concentration of the sample by solid-phase extraction, the possibility claimed by Kunkel and Wätzing (19) of avoiding comigration with endogenous plasma components and thus increasing the injection time to 15–20 s appears quite promising and merits testing, especially for GB analysis. More efforts in this direction are advisable, however, to look for improved and more sophisticated preanalytical and analytical procedures.

Because of their extensive hepatic metabolism, SU drugs of the third generation are excreted mainly in urine as hydroxylated metabolites (25, 26), and screening for drug abuse in this matrix may sometimes be complex because of the number of different oral hypoglycemic compounds commonly prescribed and the ease of availability for people willing to use these drugs covertly. Hence, despite the advantage that urinary excretion of metabolites occurs for several days after intake, we chose to look for the presence of parent drugs in serum. In addition, urine collection may be difficult in patients who sometimes have extensive psychological and social problems. Our hospitalized patients “suspected” of drug abuse are under strict control, and blood is collected along with the “suppression test” and/or whenever blood glu-

Fig. 8. Identification of TL in the serum of patient 2 by CE.
Electropherograms were obtained from the solid-phase-extracted serum (1 mL) of patient 2 (-----) and from a control serum (1 mL) enriched with 2 mg/L TL and GB (——). N-Acetyl-5-(2,3-dichlorophenylureido)benzenesulfonamide was added as internal standard (5 mg/L; ○). Dried extracts were reconstituted with 60 μL of acetonitrile:H₂O (60:40, by volume) and injected for 2 s at low pressure. Estimated concentration of the peak at the tᵣ of TL was 14.1 mg/L. The peak at tᵣ = 3.77 min did not correspond to any of the SU drugs under analysis.
cose concentrations are <2.2 mmol/L (40 mg/dL). It is worth noting, moreover, that factitious disorder often occurs after ingestion of above-therapeutic dosages of the drug.

In this report, we presented two cases of hypoglycemia after an overdose of SU drugs administered factitiously, disclosed by HPLC and confirmed by CE. However, when GB concentrations measured by HPLC were in the therapeutic range (~0.030 mg/L), CE failed to validate the result.

In conclusion, we have demonstrated that CE provides correct quantification of SU drugs in serum and can easily replace HPLC in pharmacokinetic studies (taking into account detection limits). We do not propose CE alone as a technique able to give a definitive diagnosis of drug abuse, even when coupled to diode array scanning. The availability of a method in addition to HPLC based on a different principle, however, may be pivotal in unmasking the illicit use of hypoglycemic agents. To help physicians in insulinoma diagnosis, we currently follow a multistep identification approach that includes different techniques such as reversed-phase HPLC with direct ultraviolet detection (13–15), reversed-phase HPLC with precolumn derivatization (16, 17), and CE (18–21). Only when results are still ambiguous or do not agree with biochemical testing and clinical features do we ask for confirmation by the unequivocally most specific (but less readily available) mass spectrometry.

References