Fast In Vivo Microextraction: A New Tool for Clinical Analysis

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Background: We sought to develop a technique with the potential to partly replace current methods of analysis based on blood draws. To achieve this goal, we developed an in vivo microextraction technique that is faster than conventional methods, interferes minimally with the investigated system, minimizes errors associated with sample preparation, and limits exposure to hazardous biological samples.

Methods: Solid-phase microextraction devices based on hydrophilic polypyrrole and polyethylene glycol coatings were used for direct extraction of drugs from the flowing blood of beagle dogs, over a period of 8 h. The drugs extracted on the probes were subsequently quantified by liquid chromatography coupled to tandem mass spectrometry. Two calibration strategies—external and standard on the fiber—were used to correlate the amount extracted with the in vivo concentration.

Results: Diazepam and its metabolites were successfully monitored over the course of a pharmacokinetic study, repeated 3 times on 3 beagles. The fast microextraction technique was validated by comparison with conventional plasma analysis, and a correlation factor of 0.99 was obtained. In addition to total concentrations, the method was useful for determining free drug concentrations.

Conclusions: The proposed technique has several advantages and is suitable for fast clinical analyses. This approach could be used not only for drugs, but for any other endogenous or exogenous compounds.

The need for new analytical devices for rapid medical diagnostics is growing. Point-of-care devices for measuring electrolytes, cardiac markers, and small molecules now reside in nursing stations, surgical suites, emergency rooms, and even at patient bedside. Current techniques that are applicable for in vivo analysis include microdialysis, ultrafiltration, solid-phase microextraction (SPME), sensors and sensor arrays, microfluidics, and nanotechnology (1). Although microdialysis has numerous in vivo applications (2–5), it has many problems, the most important of which are the loss of perfusion fluid and the difficulty in obtaining reliable quantitative data (6). Ultrafiltration avoids time-consuming recovery calculations (7), but the membranes are prone to clogging, and the method is not suitable for compounds that are highly bound to plasma proteins. Both ultrafiltration and microdialysis require the presence of tubing and pumps. Despite the advantages of sensors and sensor arrays, such devices are very difficult to produce, may not be suitable for complex biological samples, and are found mostly in research laboratories (1, 8, 9). Microfluidic systems remain more complicated and bulkier than a simple integrated miniature device because, in many cases, external optics, pumps, and detectors are required to control and read out signals from the chips. In addition, liquid reagent reservoirs must be incorporated into the systems; consequently, reagent stability and applicability for in vivo analysis remain problematic (1). Because of their submicrometer size, nanosensors, nanoprobes, and other nanosystems are revolutionizing the field of chemical analysis (10). However, despite their numerous advantages, nanomaterials have significant cytotoxicity, and their applicability to in vivo measurements has not been sufficiently demonstrated (11, 12). An alternative, noninvasive sampling technique is iontophoresis, which has been successfully applied to a wide variety of analytes (13, 14). Although considered safe, there are important medical issues concerning the epidermal and dermal effects of iontophoresis (15).

One of the most promising techniques for rapid sample preparation and subsequent analysis is SPME, which is based on fibers coated with biocompatible materials that

1 Nonstandard abbreviations: SPME, solid-phase microextraction; PPY, polypyrrole; PEG, polyethylene glycol; and LC-MS/MS, liquid chromatography–tandem mass spectrometry.
are exposed to the sample and then removed and analyzed. This technique causes minimal disturbances to the investigated system because no liquid and only small fractions of analytes are removed. Experimental errors and the time associated with sample transport and storage can be reduced, enabling more rapid collection of more accurate, precise analytical data (16). Numerous SPME applications have been developed for environmental, food, forensic, and clinical analyses. However, the only published procedure describing direct in vivo extraction involves lengthy extraction times (30 min), cumbersome insertion of bare SPME fibers into the vein, and manual injection into the analytical system (17).

The most widely used sampling technique based on SPME consists of exposing a small amount of extracting phase (coating) bound to a fiber to the sample for a predetermined amount of time. The number of moles of analyte (n) extracted by the coating can be calculated as

\[ n = K_{fs} \times V_f \times c_0 \]

where \( c_0 \) is the initial concentration of a given analyte in the sample, \( V_f \) is the fiber coating volume, and \( K_{fs} \) is the distribution coefficient of the analyte between the fiber coating and sample matrix (18). This equation points to the usefulness of the technique when the volume of the sample is unknown because the amount of extracted analyte is independent of the volume of the sample. In practice, there is no need to collect a defined sample before analysis because the fiber can be exposed directly to the circulating blood, ambient air, water, or other liquid sample, thereby accelerating the whole analytical process.

The amount of analyte extracted to the fiber coating is at a maximum when equilibrium is reached, thus achieving the highest sensitivity. If sensitivity is not a major concern of the analysis, the extraction time can be shortened and the fiber analyzed before equilibrium is reached. When an SPME coating that is preloaded with a standard compound is exposed to a sample matrix, desorption of the compound from the fiber occurs. The isotropy of absorption and desorption in SPME allows for the calibration of absorption by use of desorption. This is particularly important for the calibration of on-site, in situ, or in vivo analyses because control of the matrix agitation conditions is sometimes difficult, and direct addition of standard compounds into the matrix is typically not possible in these cases (19). Here we present the first application of this new calibration technique during in vivo sampling. In addition to convenient in vivo applications, SPME is proposed as a new technique for determining free concentrations of analytes (20–23). Measurement of unbound compound is necessary not only to study the effective or bioavailable quantity of a drug, but is also a way to determine the binding affinity or partition coefficient of a compound.

With the in vivo sampling method presented here, target compounds are quickly microextracted directly from their usual surroundings with the help of an SPME device (Fig. 1). This SPME device does not require wires, tubes, or pumps attached to the extraction system. Extraction time is short (30 s to 2 min), and the probes are desorbed in small volumes of solvents that are analyzed with an automated system.

**Materials and Methods**

**PREPARATION OF SPME PROBES**

In vivo sampling is much more demanding than conventional sampling. All materials must be biocompatible and sterilizable, preferably by autoclaving. As biocompatible extraction phases, polypyrrole (PPY) and polyethylene glycol (PEG) bound to C18-silica were chosen for their well-known biocompatibility (17, 24–27). PPY was synthesized by anodic oxidation of the pyrrole monomer (from Sigma/Aldrich) as described previously (17). PEG was received as a research sample from Supelco. To obtain SPME fibers, both polymers were deposited as thin layers on fine stainless steel wires (127 μm in diameter; medical grade; from Small Parts Inc.). Before deposition, the wires were properly treated to improve the stability and adherence of the coatings. PPY is a solid adsorbent whose hydrophilicity was improved by addition of triethylene glycol (28). PEG is a liquid absorbent whose extraction capacity was boosted by the addition of C18-silica, a very well known stationary phase used in liquid chromatography.

**PREPARATION OF SPME DEVICES**

The device for in vivo extraction consisted of a flexible thin wire coated with a biocompatible extraction phase and housed inside a hypodermic needle. For applications involving extractions from the veins of large laboratory animals (beagles), the total length of the device was 8 cm. A 2-inch-long (5.1 cm) hypodermic needle from BD Inter-
national was used as the outer sheath (Fig. 1). The assembly was sealed with silicone glue.

INTERFACES FOR IN VIVO APPLICATION OF SPME
The interface for experiments on beagles consisted of an in-dwelling catheter, (20-gauge; length, 1.25 inch; from BD International). The catheter was sealed with a PRN adapter (BD International), which allowed multiple sampling with SPME probes, as well as blood draws.

IN VITRO EXPERIMENTS
Extensive in vitro tests for evaluating the extraction characteristics of the fibers were performed before in vivo experiments. SPME devices were characterized for extraction time profile, desorption efficiency, limit of detection, and linear range. The influence of blood flow rate on extraction characteristics was tested with the help of an artificial circulatory system (see Fig. 1 in the online Data Supplement that accompanies this article at http://clinchem.org/content/vol52/issue4). As expected, higher flow rates led to shorter equilibration times (the period required for the analyte to reach steady-state distribution between extraction phase and sample), and lower flow rates led to longer equilibration times. Nevertheless, the total amount of drug extracted at equilibrium was the same regardless of the flow rate (29). All standard benzodiazepines were purchased from Cerilliant. Fresh dog whole blood (sterile, with EDTA as anticoagulant) was purchased from Bioreclamation. Further probe characterizations were performed as described previously by Lord et al. (17).

PROBE DESORPTION
A major limitation of previous in vivo studies was the use of an interface for manual desorption. For the present study, all probes used during a study were desorbed in parallel in plastic inserts with 20 μL of desorption solvent consisting of acetonitrile–water (75:25 by volume) containing 1 mL/L acetic acid. Lorazepam was included in the desorption solution as an internal standard (25 μg/L), to control for variation in injection volume. The probes were removed after 1 min, when desorption was complete. The resulting solution was injected automatically into a liquid chromatography–tandem mass spectrometry (LC-MS/MS) system.

LC-MS/MS ASSAY
Analyses were performed on an LC-MS/MS system consisting of a Shimadzu 10AVP liquid chromatograph with a system controller and dual binary pumps interfaced to a CTC-PAL autosampler and an MDS Sciex API 3000 tandem mass spectrometer. The assays were carried out as described previously (17). In addition, the diazepam-d₅ transition from m/z 290.4 to 198.4 was monitored.

ANIMAL EXPERIMENTS
All experimental procedures on dogs were approved by the Animal Care Committees at the Universities of Guelph and Waterloo. Generally, the experiments were performed as described previously (17). The most notable differences consisted of a different sampling schedule, use of single-use devices based on hypodermic needles, and use of a single catheter for both SPME and conventional blood draws.

Drug concentrations were monitored for 8 h after dosing with diazepam. For each time point, the probes were in place for 2 min before the stated analysis time, and blood draws were performed immediately after removal of the probes. For a selected number of time points (at 1 and 2 h), the blood draw was followed by insertion of a supplementary SPME probe preloaded with diazepam-d₅. These preloaded probes were exposed to the blood flow for only 30 s (standard-on-the-fiber approach). At each time point, 1 sterile SPME device was placed through the catheter into the cephalic vein so that only the coated portion of the wire was exposed to the venous Fig. 2. In vitro calibration for diazepam (†), nordiazepam (□), and oxazepam (▲) from dog whole blood incubated with 10% CO₂.

A 6-point calibration (n = 3) from 5 to 750 μg/L is shown.
blood. To insert the probes, the PRN adapter was pierced with the SPME device. Subsequently, the device plunger was depressed all the way to the back of the needle. The length of the device was chosen so that the coated wire was completely exposed to the blood flow when the plunger was fully depressed. All probes were single use. In previous experiments (17), the venous blood could flow out of the vein during insertion and removal of probes. The design of the new devices (Fig. 1) prevents leaking of blood. For comparison and validation pur-

![Graphs showing pharmacokinetic profiles](image)

*Fig. 3. Diazepam (A), nordiazepam (B), and oxazepam (C) pharmacokinetic profiles from 3 studies on 3 dogs (n = 9).*  
•, in vivo SPME from whole blood; ■, conventional analysis with plasma; ▲, in vivo standard on the fiber with PPY probes; +, in vivo standard on the fiber with PEG probes. For easier visualization, the time axis is shifted, with 10 min for plasma analysis and 20 min for the standard-on-the-fiber approach.
poses, blood draws were taken from the same catheter after each sampling with SPME.

CONVENTIONAL PLASMA ANALYSIS
Plasma was prepared and analyzed as described previously (17), aside from the concentration of internal standard (5 μg/L lorazepam) and the volume of sample injected in the chromatographic system (20 μL). The linear range was 0.1–1000 μg/L.

Results and Discussion
In previous studies, bare SPME wires were manually introduced into the vein through a catheter. In the present study, the wire with extraction phase was completely enclosed in a device based on a hypodermic needle. The new SPME devices could be positioned either directly in the vein when only a few determinations were required or through an in-dwelling catheter when several successive analyses were required. For the LC-MS/MS quantification of diazepam, nordiazepam, and oxazepam, the chromatographic peaks were sharp, and no interference from the blood matrix was observed (see Fig. 2 in the online Data Supplement).

The microextraction technique caused minimal disturbances to the investigated systems as no liquid and only small fractions of analytes were removed. Consequently, the investigated system was minimally disturbed, and additional information, such as the free concentration, could be obtained.

In many clinical situations, measurement of the total drug concentration does not provide the needed information concerning the free concentration of drug in plasma that is available for pharmacodynamic action; the present technique addresses this necessity. With SPME, the concentration of free analyte is determined by calibration against buffer or protein-free plasma (22). Nevertheless, the total concentration can be determined as well by in vitro calibration against whole blood incubated with 10% CO2 (Fig. 2) (17). Because diazepam and its metabolites are rather highly bound to plasma proteins (~90%), only 10% of analytes are available for extraction and detection with SPME. Nevertheless, method sensitivity was suitable for the current study. If the analytes are highly bound to plasma proteins (>99.9%) and sensitivity is an issue, extraction phases with high specificity and affinity, such as molecularly imprinted polymers or antibodies, should be used.

For reliable quantification, the natural variability of blood flow rate must be taken into account. To this end, we used 2 strategies: calibration by equilibrium extraction and kinetic calibration (with a standard compound preloaded on the fiber) (19). In SPME, the usual approach consists of allowing the fiber to stay in contact with the sample until equilibrium is reached. This method offers independence of blood flow (once equilibrium has been reached), good reproducibility, and maximum sensitivity, particularly at low concentrations. However, it can be applied for in vivo determinations only when the equilibration time is short enough to allow monitoring of rapid changes in the concentrations of target compounds.

On the other hand, the recently developed method of kinetic calibration based on standards preloaded on SPME fibers offers the possibility of obtaining reliable quantitative data even when the blood flow rate is unknown and the fiber is only briefly exposed to the sample. This calibration approach is based on the isotropy of adsorption and desorption from the SPME fiber: while the target compound is extracted from the sample, the standard loaded on the fiber diffuses into the sample. The amount of standard remaining on the fiber after a certain period of time depends on the agitation conditions in the sample, as is the case with the amount of analyte that is adsorbed. The experimental procedure consists of 2 steps: loading of the probe with a known amount of standard, followed by short exposure to the sample (before equilibrium is reached). Quantification of the standard that remains on the fiber and of the target compound extracted permits calculation of analyte concentration in the sample (19).

In the case of calibration by equilibrium extraction, care must be taken to ensure that equilibrium is reached during sampling. This is usually achieved by determination of equilibration time in the least favorable situation: a very low flow rate or a static sample. In vitro simulations based on an artificial circulatory system and incubator indicated equilibration times of 2 min for PPY and 30 min for PEG. The significant difference in equilibration time is a consequence of a major contrast in their structure: PPY is a thin (10 μm) solid adsorbent with low capacity, whereas PEG loaded with C18-silica is a somewhat thicker (60 μm) absorbent coating with high capacity. Because of their short response time, the PPY fibers can be used with both methods of calibration. On the other hand, the high capacity and long equilibration time of PEG fibers makes them better suited for the standard-on-the-fiber approach. We investigated the sensitivity, reproducibility, and linear range of the assay by in vitro analysis of buffer and dog whole blood to which drug had been added in a series of concentrations. All in vitro samples were incubated in 10% CO2 to create experimental conditions similar to in vivo conditions and to generate accurate calibrations. The linear range for diazepam, nordiazepam, and oxazepam in whole blood was 3–750 μg/L (total concentration), corresponding to 0.18–46 μg/L for the free concentration.

The rapid in vivo microextraction methodology was evaluated during studies of diazepam pharmacokinetics in beagles. Two of the main metabolites of diazepam, nordiazepam and oxazepam, were monitored as well. PPY probes were exposed to the flowing blood for 2 min in the case of equilibrium extraction and for 30 s for kinetic calibration. PEG probes were used only with kinetic calibration and were exposed for 1 min. Diazepam-d5 was used as the standard on the fiber for kinetic calibrations.
Because of the short sampling time of the new SPME devices, a single investigator could perform 3 pharmacokinetic studies per day (in parallel). It was also possible to draw the blood through the same catheter that was used for probe sampling, which reduced the stress for the experimental animals. Visual inspection of the sampling procedures indicated that the beagles felt more uncomfortable during conventional blood draws than during SPME sampling.

Immediately after extraction, the probes were briefly rinsed with water and stored on dry ice (−20 °C) until the next day. The investigated compounds are known to be stable on the fibers for at least 24 h (17). The maximum total time required to prepare a single sample for LC-MS/MS analysis was 3 min in the case of SPME and 90 min for conventional plasma analysis. Parallel processing in 96-well plates can significantly decrease the total amount of time required for analysis of multiple samples.

The diazepam, nordiazepam, and oxazepam pharmacokinetic profiles obtained with the probes, which measured concentrations in whole blood, compared with the results of conventional analyses from plasma are shown in Fig. 3. Fig 3 presents the data obtained with both equilibrium and kinetic calibration. Because of the distribution of drugs into blood cells, the ratio between plasma and whole blood concentrations can be anywhere from 1.35 to 2.22 (30). Accordingly, no correction factor was used for the comparative graphs. Nevertheless, all 3 compounds showed excellent correlation between the values obtained with SPME and conventional analysis (Table 1).

The diazepam concentration showed a triple-exponential decrease, with similar half-lives in both analysis methods. The same was true for nordiazepam and oxazepam, which showed single- and double-exponential elimination, respectively (Table 1). All pharmacokinetic profiles are in agreement with literature values (17, 31). Kinetic calibration was performed only for the data points collected at 1 and 2 h, as this was the first in vivo application of standards on the SPME fiber. On the other hand, the well-known equilibrium extraction was applied for all data points.

An important strength of this in vivo assay is the ability to determine free drug concentrations, which are of pharmacologic significance (22). The free-concentration profiles for all 3 compounds in dog whole blood (Fig. 4) revealed rapid in vivo conversion of diazepam to oxazepam, with similar values for their free concentrations. On the other hand, the free concentration of nordiazepam remained almost constant for the first hour, followed by a single-exponential decrease.

The free concentration was determined by calibration with standard solutions of benzodiazepines in phosphate-buffered saline (pH 7.4), and the total concentration was determined by calibration with standard solutions in whole blood. Whereas the phosphate-buffered saline solution has a well-known and reproducible composition, whole blood used for calibration purposes may have a

Table 1. Comparison of pharmacokinetic data obtained with SPME vs conventional sample preparation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Diazepam&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Nordiazepam&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Oxazepam&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correlation (r)</td>
<td>0.99</td>
<td>0.97</td>
<td>0.99</td>
</tr>
<tr>
<td>Half life, h</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>SPME</td>
<td>( t_{1/2} = 0.12 )</td>
<td>( t_{1/2} = 0.16 )</td>
<td></td>
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<tr>
<td></td>
<td>( t_{1/2} = 0.41 )</td>
<td>( t_{1/2} = 0.50 )</td>
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<tr>
<td></td>
<td>( t_{1/2} = 2.37 )</td>
<td>( t_{1/2} = 2.26 )</td>
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<tr>
<td></td>
<td>( t_{1/2} = 5.03 )</td>
<td>( t_{1/2} = 1.01 )</td>
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<tr>
<td></td>
<td>( t_{1/2} = 4.77 )</td>
<td>( t_{1/2} = 1.57 )</td>
<td></td>
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<tr>
<td>Conventional</td>
<td>( t_{1/2} = 0.10 )</td>
<td>( t_{1/2} = 0.12 )</td>
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<td></td>
<td>( t_{1/2} = 0.50 )</td>
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<td>( t_{1/2} = 2.37 )</td>
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<td>( t_{1/2} = 4.77 )</td>
<td>( t_{1/2} = 1.57 )</td>
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</table>

<sup>a</sup> Profile shows triple-exponential elimination.
<sup>b</sup> Profile shows single-exponential elimination.
<sup>c</sup> Profile shows double-exponential elimination.

![Figure 4](image-url)  
**Fig. 4.** Comparative pharmacokinetic profiles for diazepam (●), nordiazepam (□), and oxazepam (▲; free concentrations).
different composition than in vivo blood. The values for the in vivo total concentration were calculated assuming that the blood used for calibration has the same binding properties (for diazepam and its metabolites) as the in vivo blood, which is usually the case for healthy individuals. The results for total concentration are meaningful when drug binding does not change significantly during analysis. Conversely, reliable measurements of the free concentration, a valuable marker, can be obtained even when the concentration of plasma proteins changes because the amount of analyte extracted by SPME is inherently related to the free concentration.

In this first application of fast microextraction and kinetic calibration for in vivo analysis, sampling devices based on hypodermic needles with SPME fibers were developed and successfully used for investigation of free and total concentrations in whole blood. The main advantages of this approach are reduced exposure to blood, rapid sample preparation, and considerate treatment of laboratory animals. Fewer animals can be used when fast microextraction is applied in pharmacokinetic studies, which reduces the interanimal variation of experimental results. More data points may be obtained for each animal. Additionally, sampling can be performed simultaneously at multiple sites in one animal without the risk of exsanguination. The technique has several advantages over current methods: it is much faster, no biological sample processing is required, supplementary variables are revealed, and the analysis of extracted compounds can be performed with highly specific instruments, such as LC-MS/MS. Further developments of this technology are underway, including applications in soft tissues, automation of the sampling process, and use of highly specific extraction phases, such as molecularly imprinted polymers or antibodies.

In our study, monitoring of free and total circulating drug concentrations was performed in beagles. We believe that this technique can be transferred directly to humans because biocompatible materials were used and the dimensions of the device are appropriate for human veins. However, more tests are required to ensure the safety of the method.

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References


