Application of Electron Spin Resonance to Determination of Serum Drug Concentrations

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Displacement of spin-labeled drugs from drug-specific antibodies in human serum can be measured by use of electron spin resonance. This "spin immunoassay" has advantages of speed, simplicity, and small volume requirements over other standard techniques such as gas-liquid, liquid-liquid, or thin-layer chromatography. Unlike radioimmunoassay, there is no need to separate free and bound label and there are no radiation hazards. We present clinical and experimental data showing how this method can be applied to the routine determination of morphine and diphenylhydantoin concentrations in serum.

Additional Keyphrases: spin-labeling vs. radiolabeling • diphenylhydantoin • morphine • inter-method comparison • toxicology

The ideal clinical laboratory technique for determining chemicals in biological fluids should be simple, specific, readily automated, and applicable to a wide range of compounds. A significant advance in drug assays was the development of radioimmunoassay. Although this elegant technique meets many of these criteria, there are several ways in which it may be improved. Most importantly, in many radioimmunoassays the metabolites of a drug may be recognized by the antibody, resulting in a falsely elevated value for the concentration of the parent compound. This problem, inherent for all types of immunoassays, must be carefully evaluated for each individual assay.

Another important limitation of the radioimmunoassay is the necessity to physically separate free and antibody-bound label. This separation requires time and materials, increasing both costs and the possibility of introducing errors.

In this report we describe our experiences with an alternative immunoassay technique, the spin immunoassay, which is faster and simpler than other immunoassays.

In the spin immunoassay, drugs are labeled with a stable nitroxide free radical. The electron spin resonance (ESR) spectra of nitroxide radicals demonstrate anisotropy, both of the hyperfine coupling with the nitrogen and of the g value. When the nitroxide-labeled drug is bound to the drug-specific antibody ("immobilized"), the nitroxide tumbles in solution at rates that are slow relative to their hyperfine frequencies (~40 MHz). The ESR spectrum then appears as a broad envelope of lines resulting from the summing of different signals from molecules in all possible orientations relative to the magnetic field. This broadened, weak ESR signal represents the background value for the assay. Antibody-bound, immobilized drug in the biological fluid is competitively displaced by free unlabeled drug. The displaced labeled drug now tumbles rapidly in solution, causing averaging of the magnetic field positions, and yields three sharp, distinct peaks in the ESR spectrum. This characteristic three-line spectrum for nitroxide radicals arises from the isotropic nitrogen hyperfine interactions. The amplitude of these peaks is directly proportional to the number of spin-labeled drug molecules displaced from the antibody. After the background ESR signal has been subtracted, the original concentration of free unlabeled drug in the biological fluid can be estimated.

We describe here the application of this technique to the determination in serum of some clinically important drugs. Further, we have investigated the applicability of the spin resonance technique for the estimation of the binding of a number of drugs to serum proteins.

Methods

Instrumentation and drug analysis. All ESR determinations were performed on a Varian E-4 spectrometer utilizing either glass capillary tubes graduated to a volume of 50 µl ("FRAT" disposable microcapillaries, Syva Corp., Palo Alto, Calif. 93404) or the Varian aqueous solution sample cell (Model E-248). ESR settings varied with the individual experiments. Samples were prepared for ESR examination by adding 10 µl of the spin-labeled drug/antibody complex to 40 µl of the appropriate drug-containing solution (water, urine, or serum) for determination in the capillary tube, or 10 µl of the complex to 300 µl of drug-

1 The g factor is an empirical quantity, characteristic of the molecule in which the electron is located.
containing solution for determination in the aqueous solution cell. Incubation time for all samples was 60 min, as previously described (1).

Thin-layer chromatography. Serum samples containing [14C]diphenylhydantoin were extracted with chloroform/methanol (1/1 by vol), evaporated under reduced pressure, and the residues were chromatographed on silica gel GF plates with chloroform/methanol (9/1 by vol) as the developing solvent. Thin-layer chromatography of [N-methyl-14C]morphine was performed in a CCl4/butanol/methanol/NH4OH solvent system (40/30/30/2 by vol). We had great difficulty in extracting morphine from rabbit serum, as attempted by several methods (2, 3). Finally, the serum containing [14C]morphine (plus metabolite) was ultrafiltered through a Diaflo membrane (UM-10; Amicon Corp., Lexington, Mass. 02173) at 483 kPa (70 psi). The filtrate was lyophylized and redissolved in a small volume of methanol, which was applied directly to the chromatographic plate. The plates were scanned in a Packard scanner and then scraped in segments, 0.5 cm wide. These scrapings were counted in 10 ml of “Aquasol” (New England Nuclear, Boston, Mass. 02118) in a Beckman LSC-1000 scintillation counter.

Ultrafiltration. Triplicate 5-ml aliquots of fresh human sera to which diphenylhydantoin or morphine had been added (10 µg/ml, plus 1 µCi of [14C]diphenylhydantoin or [N-methyl-14C]morphine) were ultrafiltered through a Diaflo membrane (QM 10; Amicon Corp.) at 483 kPa. Aliquots, 0.1 ml, of the filtrate were solubilized in “Aquasol” and the radioactivity was determined in a Beckman LSC-1000 scintillation counter. When physiological saline was used instead of serum, the percentage of nonspecific binding to the ultrafiltration membrane was found to be 19% for diphenylhydantoin and 12% for morphine. The percentage of protein binding of diphenylhydantoin or morphine was corrected for this membrane effect.

Equilibrium dialysis. Equilibrium dialysis was performed in triplicate by dialyzing 5 ml of pooled human serum against 50 ml of saline (0.15 mol/liter) that contained 10 µg of diphenylhydantoin per ml, plus 1 µCi of [14C]diphenylhydantoin. After 24 h the difference in [14C]diphenylhydantoin activity in the serum and in the dialysate was determined by scintillation counting.

Determination of diphenylhydantoin by gas-liquid chromatography. The serum concentration of diphenylhydantoin was determined essentially by the method of Kupferburg (3). In this method 5-(4'-methylphenyl)-5-phenylhydantoin was added to the serum (1 ml) as an internal standard, to give a final concentration of 50 µg/ml. This was followed by the addition of 1 ml of potassium phosphate buffer (0.25 mol/liter, pH 7.4). The drug was extracted into 6 ml of ethylene dichloride, 5 ml of hexane was added, and the organic phase was extracted with 4.5 ml of K2HPO4 (0.2 mol/liter). The 3.5-ml aqueous phase was acidified with 0.3 ml of HCl (5 mol/liter) and finally extracted into 10 ml of ethylene dichloride. The extract was dried in a stream of nitrogen and redissolved in 50 µl of a 0.1 mol/liter solution of trimethylammonium hydroxide in methanol. The mixture was then injected into a Perkin-Elmer Model 881 gas-liquid chromatograph. The 180-cm glass column was packed with 3% OV-1 on “Gas Chrom Q” (60–80 mesh; Applied Science Laboratories, State College, Pa. 16801). The carrier gas was argon at 414 kPa (15 psi). The oven temperature was programmed from 150–200 °C at 10 °C/min after 5 min at the initial temperature. The peaks were detected with a flame ionization detector and the areas integrated with an Autolab 6500-2 digital integrator (Spectra-Physics, Mountain View, Calif. 94043).

Preparation of drug-specific antibodies and spin-labeled drugs. A detailed description of antibody preparation and purification and of the synthesis of spin-labeled diphenylhydantoin and morphine appears elsewhere (5, 6). Briefly, the drug-specific antibodies were isolated from sera of sheep that had received periodic injections of drug/bovine serum albumin conjugate emulsified in complete Freund’s adjuvant. After preparation of the antisera, binding site concentrations and association constant were determined by titration with spin-labeled drug. The diphenylhydantoin antibodies used in these experiments had a concentration of 1.9 × 10−4 mol/liter in binding sites and an association constant of 4.6 × 106 liter/mol. This antibody was diluted with borate buffer (0.4 mol/liter; pH 8.0) to a concentration of 1.8 × 10−5 mol/liter in binding sites. The morphine antibody had a concentration of binding sites of 2.4 × 10−5 mol/liter and an association constant of 1.7 × 107 liter/mol.

Figure 1 shows the molecular structures of the spin-labeled morphine and diphenylhydantoin. To prepare the spin-labeled drug/antibody complex, the spin-labeled drugs were dissolved in ethanol and diluted in distilled water to a final concentration such that when 5 µl of the antibody solution was combined with 5 µl of the spin-labeled drug solution a slight excess of spin-labeled drug was present. This is to assure complete saturation of the antibody binding sites. This mixture was then added to either 50 µl or 300 µl of drug-containing solution.

Binding of spin-labeled drugs to serum proteins. Binding of the spin-labeled diphenylhydantoin or spin-labeled morphine to serum proteins was assessed by adding an aliquot of the spin-labeled compound to human serum containing 36.5 µmol of diphenylhydantoin per liter or 0.30 µmol of morphine per liter. The amplitude to the low-field signal in the serum was compared to the amplitude of this peak in water. The decreased signal observed in the presence of serum is due to the binding of the spin-labeled drug to the serum proteins and the resulting immobilization of the free radical species (1).

Metabolism of diphenylhydantoin and morphine in rabbits. [4-14C]Diphenylhydantoin (New England
Nuclear, lot No. 648-298, specific activity 5.21 Ci/mol) was dissolved in saline and diluted with unlabeled sodium diphenylhydantoin to a final concentration of 25 mg/ml (5 mCi/liter). [N-methyl-14C]Morphine (Amersham-Searle Corp., Arlington Heights, Ill. 60005) had a specific activity of 57 Ci/mol (150 mCi/g, No. CFA 363, lot 15). This dosing solution was prepared in saline to a final concentration of 50 mg/ml with a specific activity of 70 mCi/liter. We injected 25 mg of the diphenylhydantoin solution or 10 mg of the morphine per kilogram body weight into the marginal ear vein of a 4-kg female New Zealand white rabbit. Blood samples were drawn from the opposite ear vein at various times after dosing. The serum was separated and immediately frozen for later analysis. Aliquots of serum (0.10 ml) were dissolved in 10 ml of “Aquasol” scintillator fluid and counted in a Beckman LSC-1000 counter.

Diphenylhydantoin specimen. Sera from patients receiving diphenylhydantoin for seizure control were obtained from the clinical services, V.A. Hospital, Minneapolis, Minn. 55417. The spin immunoassay determinations for diphenylhydantoin concentrations were compared to the gas–liquid chromatography method determinations.

Results

In the original studies of Leute et al. (4, 6), the spin immunoassay technique was established for the identification of opiate narcotics in urine and saliva, a procedure that simply requires mixing 20 µl of test solution with 10 µl of the spin-labeled drug/antibody complex and, after a 15–30 min equilibration period, performing a 1-min scan on the ESR spectrometer. In developing this method for determining drugs in serum, we observed several differences in the interaction of the spin-labeled drug/antibody complex with free drug.

The equilibration time required to give maximum signal for the highest concentrations of morphine in sera was 60–90 min (Figure 2). This longer period as compared to urine or saliva may be attributed to the competitive interaction of the spin-labeled and free drug, not only for the antibody but also for the non-specific binding sites on the serum proteins. Binding of a small portion of the displaced, spin-labeled drug by the serum proteins results in a smaller ESR response for a particular drug concentration in serum as compared to that same concentration in water.

However, the background signal from the spin-labeled drug/antibody complex alone also decreases. When the appropriate background (“blank”) signal is subtracted from the ESR response observed for a serum sample containing the drug, there is an increase in total sensitivity as compared to urine (Figure 3). A total peak-to-peak difference of 10 units (one ESR chart unit equals 2 mm) is taken as the lower limit of detection. When 50-µl capillary tubes are used, the sensitivity of the assay is increased in serum by about 10-fold as compared to urine (Figure 4). A further increase in sensitivity was obtained by using a quartz aqueous solution cell (Figure 4). With this cell there is a total sample volume in the spectrometer cavity of 300 µl as compared to the 50-µl volume for the capillary tubes. This increased sensitivity results from the fact that the ESR spectrometers...
Thus, drugs plications for power. The final application of diphenylhydantoin gives a sixfold decrease in the minimal detectable morphine concentration to 35 nmol/liter. This limit of assay sensitivity agrees quite well with the instrumental limit of detection of nitroxide-labeled drug, 20 nmol/liter (1).

In a subsequent series of studies we examined the application of this technique to the determination of diphenylhydantoin. The assay sensitivity required for the routine clinical monitoring of diphenylhydantoin in serum can be achieved simply with the 50-μl sample volume and a relatively low microwave power of 12 mW (Figure 5). The relatively high degree of serum binding of diphenylhydantoin results in a virtual disappearance of the background resonance signal in this assay. Thus, as was the case for morphine, the sensitivity of the diphenylhydantoin assay can quickly and conveniently be increased by about 100-fold by increasing the sample volume and microwave power. This high sensitivity, although not required for routine clinical situations, would have obvious applications to forensic and experimental medicine.

To assess the reliability and validity of the spin immunoadsay technique for the determination of drugs in serum and for its application in monitoring their concentrations, we gave a female rabbit either [N-methyl-14C]morphine (10 mg/kg, i.v.) or 5,5-diphenylhydantoin (25 mg/kg, i.v.). Serum drug concentrations were determined at various time intervals from 15 min to 24 h after drug administration by liquid scintillation counting and the spin immunoadsay technique for morphine and also gas-liquid chromatography for diphenylhydantoin.

In the case of morphine the distribution phase was complete in 90 min, after which the disappearance was linear up to 6 h (Figure 6). Not only was there good agreement between the half-lives obtained by both methods for this fast phase of disappearance ($t_{1/2}$ (14C) = 1.9 h, $t_{1/2}$ (ESR) = 1.6 h), but also there was excellent agreement between the concentrations at each sample point. The sample drawn at 24 h represented the second, slow phase of morphine disap-

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9 Power saturation, i.e., decreased ESR response with increasing microwave power, begins to occur with nitroxide-labeled drugs at powers greater than 50 mW.
pearance \((t_{1/2} = 16 \text{ h})\) and the agreement between the results by the two methods was again excellent. A sample drawn at 48 h yielded a serum morphine concentration of \(40 \pm 3 \mu \text{g/liter} \) (\(\pm \text{ SEM for triplicate determinations} \)) by the ESR method. However, at this point less than 0.05% of the original \(^{14}\text{C}\) activity remained. This small residual \(^{14}\text{C}\) activity was less than twice background and thus comparison between the two techniques was impossible.

In the case of diphenylhydantoin, the higher dosage (25 mg of diphenylhydantoin per kilogram vs. 10 mg of morphine per kilogram) was selected to produce serum diphenylhydantoin concentrations similar to those found in man after therapeutic doses of diphenylhydantoin \((8, 9)\), that is, 1.0 to 40.0 \(\mu \text{g} \) of diphenylhydantoin per milliliter of serum. The agreement among the \(^{14}\text{C}\) activities, the gas–liquid chromatography determinations and the spin immunoaassay values were excellent; the \(t_{1/2}\) for this linear phase of disappearance was 4.0 h. At concentrations of diphenylhydantoin in serum greater than 40 mg/liter, the spin immunoaassay tended to give slightly higher values than those from \(^{14}\text{C}\) activity or gas–liquid chromatography. This discrepancy disappeared when these samples were diluted with equal volumes of serum containing no diphenylhydantoin.

These combined data for morphine and diphenylhydantoin determinations in serum also indicate a high degree of intra-assay precision for the spin immunoaassay. The average intra-assay coefficient of variation for triplicate determinations was 6%.

Thin-layer chromatography of rabbit serum extracts indicated that only 4% of the \(^{14}\text{C}\) activity of the diphenylhydantoin was present as the hydroxylated metabolite and only about 10% of the morphine was present as the glucuronide metabolite. Thus, the \(^{14}\text{C}\) activity gives a reasonably good estimate of the true concentrations of these drugs in serum. Both metabolites react in this immunoaassay system similarly to the parent compound \((5, 6)\).

The question of cross-reactivity in the spin immunoaassay has been dealt with in detail previously \((5, 6)\). Few non-opiate drugs cross react with the morphine assay and those that do give only weak signals at very high concentrations. Barbiturates, amphetamines, and cocaine do not cross react. Likewise, antihistamines, among which is oxymetazoline hydrochloride which gives a false positive for morphine in the standard clinical thin-layer chromatography assay for morphine, were not detected even at a concentration of \(10^{-3} \text{ mol/liter} \) in the morphine spin immunoaassay system.

In the diphenylhydantoin spin immunoaassay, of eight drugs commonly used in conjunction with diphenylhydantoin for seizure control (glutethimide, diazepam, chlordiazepoxide, mesantoin, ethosuximide, primidone, and phenobarbital), only primidone was recognized by the antibody to diphenylhydantoin, a cross-recognition that we are now investigating. Because antibodies produced in different animal species with different conjugates are known to have different reactivities toward the same compound, we are trying to develop a different antibody source to circumvent this problem.

Although the drug metabolites, morphine glucuronide and \(5-(4'-\text{hydroxyphenyl})-5\)-phenylhydantoin are fully recognized by the antibody as the parent drug, these metabolites are rapidly excreted and, therefore, do not represent a large source of error in the serum assays. In our hands, only 4% of the total diphenylhydantoin was present in the serum as the \(5-(4'-\text{hydroxyphenyl})-5\)-phenylhydantoin metabolite and a maximum of 12% of the total morphine was present as the glucuronide 6 h after injection of the drug.

We evaluated the clinical reliability of this technique for determination of diphenylhydantoin concentrations in a group of 28 patients, chosen without conscious bias, who were receiving diphenylhydantoin for seizure control. Their sera were assayed for diphenylhydantoin by spin immunoaassay and by gas–liquid chromatography, and the results agreed well \((r = 0.97, P < 0.001)\). Of the 28 patients, four were found by both methods to have diphenylhydantoin concentrations exceeding 25 mg/liter, which meant that they were receiving too much of the drug. Ten patients had <5 mg of diphenylhydantoin per liter of serum by both methods and required an appropriate upward adjustment of diphenylhydantoin dosage. The remaining 14 patients were adequately maintained \((8–18 \text{ mg of diphenylhydantoin per liter})\). Not only was there excellent clinical and statistical agreement, but also the regression line for the comparison of the two methods had a slope that was not significantly different from 1.0 (slope ±1 SD = 0.94 ± 0.04), indicating that the spin immunoaassay measures diphenylhydantoin as accurately and precisely as does gas–liquid chromatography.

The decreased ESR signal amplitude that results from the binding of the spin-labeled drug to serum proteins can be quantitated by comparing the signal amplitude in serum to that for the same concentration of spin-labeled drug in water. The data (Table 1)

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<tr>
<th>Drug</th>
<th>Equilibrium dialysis</th>
<th>Ultrafiltration</th>
<th>ESR (^b)</th>
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<tbody>
<tr>
<td>Morphine</td>
<td>35.4 ± 1.4</td>
<td>30.5 ± 0.4</td>
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<tr>
<td>Diphenylhydantoin</td>
<td>87.9 ± 1.0</td>
<td>88.7 ± 1.2</td>
<td>61.0 ± 1.9</td>
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\(^a\) Data represent mean ± SEM for three separate determinations at a morphine concentration of 0.30 \(\mu\text{mol/liter} \) and diphenylhydantoin concentration of 36.5 \(\mu\text{mol/liter} \).

\(^b\) ESR settings: scan rate, 5 G/min; time constant 3 s; field set, 3376 G (uncalibrated); modulation amplitude, 4 G; modulation frequency, 100 kHz; microwave power, 12 mW; microwave frequency, 9.537 GHz.
These spin-labeled diphenylhydantoin noassay. simpler, protein-binding maintained characteristics bound molecule labeled characteristics results indicate that this method gives an accurate estimate of the percentage binding of morphine to serum proteins as compared to the standard method of ultrafiltration. However, the percentage of the spin-labeled diphenylhydantoin bound did not agree well with the results of ultrafiltration and equilibrium dialysis. This difference in uniformity of binding of the two spin-labeled drugs may be explained by the charge characteristics of the two ligands. In the case of spin-labeled morphine, the charge of the spin-labeled molecule is essentially unaltered as compared to nonlabeled morphine. On the other hand, spin-labeled diphenylhydantoin has a positive charge on the amine linkage bound to the hydantoin ring and therefore is more cationic and hydrophilic; hence less is bound to the acid-binding sites on serum proteins. These results would suggest that the exact electronic characteristics of the parent drug molecule must be maintained during synthesis if identical serum protein-binding characteristics are to be retained in the spin-labeled product.

Discussion

The use of spin-labeled drugs in place of radiolabeled drugs appears to be a potentially useful immunoassay technique. The extremely small sample volume (50 μl) required, the simplicity of sample preparation, the rapidity of instrumental determination, and avoidance of the need to separate free and bound label all help to make the spin immunoassay faster, simpler, and less prone to error than the radioimmunoassay.

The limit of detection, 35 nmol of drug per liter of serum, places this technique well within the normal range for a large number of commonly used therapeutic compounds, many of which have therapeutic concentrations in serum of 100 nmol to 100 μmol per liter (10). For example, this lower limit of detection is 100-fold less than the sensitivity required for routine clinical monitoring of serum diphenylhydantoin (1–50 mg/liter). Therefore, this technique appears to be readily adaptable for long-term monitoring of drug disappearance and kinetics in man or in laboratory animals. Furthermore, because serum volumes required are comparable to those used in the radioimmunoassays, this technique would be suitable for clinical and experimental pharmacokinetic studies involving neonates.

The mechanical configuration of the microwave cavity in the ESR spectrometer should readily facilitate complete automation of this technique. A continuous flow-through system is now under development, which combines an AutoAnalyzer (Technicon) mixing system to deliver 10 μl of a pre-mixed spin-labeled drug/antibody complex into 40 μl of a serum sample. The sample then flows through a coiled tube for the 60-min equilibration period and then into the quartz sample cell in the ESR cavity. Because there is an air bubble between the samples, the 30-s spectrum can be initiated by means of a photo switch. The signal amplitude is then recorded, quantitated, and stored in an interfaced computer. Such a procedure should permit determination of multiple sera samples at a low cost.

Finally, with a large variety of spin-labeled drugs and corresponding antibodies, it is attractive to envision the mass drug-screening potential of this automated assay, with use of only 2–3 ml of patients’ sera. Each 50-μl aliquot of sera could be mixed with a different spin-labeled drug/antibody complex in the system described above and a quantitative profile obtained of the drug content of the sample. The clinical and medical/legal applications of such a rapid screening capability are obvious.

References