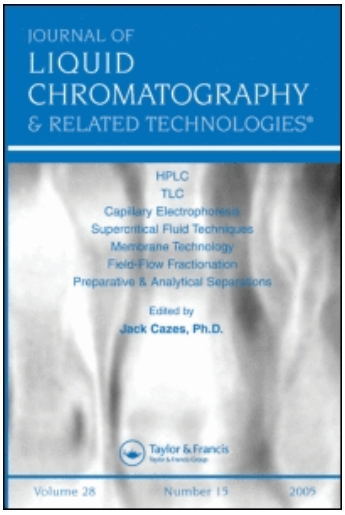


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# Hypnotic-Sedative Screen by High Performance Liquid Chromatography

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# HYPNOTIC-SEDATIVE SCREEN BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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## ABSTRACT

In this procedure some of the most commonly abused sedatives and hypnotics can be identified and quantitated using 100 ul of serum. Ethosuximide, primidone, methyprylon, phenobarbital, butabarbital, butalbital, glutethimide, mephobarbital, phenytoin, carbamazepine, secobarbital, phensuximide, pentobarbital, amobarbital, and methaqualone are extracted into a chloroform-ethanol solvent containing either 5-(p-methylphenyl)-5-phenylhydantoin or hexobarbital as an internal standard, evaporated to dryness, reconstituted with mobile phase and injected into a high performance liquid chromatograph in conjunction with a radial compression separation system. Peak heights are measured at 195 nm and 254 nm and sensitivity for all drugs is one μg/ml. Day to day precision obtained CV's ranging from 4.5 to 10.4%.

## INTRODUCTION

High pressure liquid chromatography has been increasingly used for separation and quantitation of multiple drugs simultaneously in biological fluids (1-3). Kabra and associates (4) published an HPLC technique that could simultaneously measure 12 common sedatives using acetonitrile precipitation of protein and a heated reverse phase μ-Bondapak rigid column for separation.

We were interested in modifying their procedure to the recently introduced Radial Compression Separation System (RCSS - Waters Associates Inc., Milford, Ma). The RCSS utilizes a compression chamber to radially compress a flexible-walled cartridge containing microparticle spherical material. The RCSS offers several advantages over the rigid-walled columns (5-6). The columns are essentially nonvoiding allowing rapid separation and short analysis time. The spherical particles and radial compression allows higher flow rates without damage due to elevated back pressure and thus regeneration is easier and faster than using the rigid-walled columns. Finally, changeover of columns and equilibration times of the RCSS is faster than using rigid-walled columns.



In the present study an assay method is described where 15 of the most common abused anticonvulsants, sedatives and hynotics can be separated and quantitated in 100 µl of serum using the new radial compression separation system. Ethosuximide, primidone, methyprylon, phenobarbital, butabarbital, butalbital, glutethimide, mephobarbital, phenytoin, carbamazepine and secobarbital are extracted into a chloroform-ethanol solvent containing 5-(p-methylphenyl)-5-phenylhydantoin as an internal standard. Phensuximide, pentobarbital, amobarbital and methaqualone are extracted into a chloroform-ethanol solvent containing hexobarbital as an internal standard. Aliquots of the solvents are evaporated to dryness, reconstituted with mobile phase and

injected into a high pressure liquid chromatograph in conjunction with a radial compression separation system for separation and subsequent quantitation at 195 nm (254 nm for carbamazepine and methaqualone).

## MATERIALS

HPLC was carried out on a Waters Associates ALC-GPC 200 series instrument (Waters Associates Inc., Milford, MA) equipped with a U6K injector, Model 6000A solvent delivery system, Model 440 UV detector with a 254 nm filter (0.005 aufs), Model 450 variable wavelength UV detector at 195 nm (0.1 aufs) and Omniscribe (Houston Instruments, Houston, TX) dual pen recorder.

Chromatographic separation was accomplished with a Waters Associates Radial Compression Separation System (RCSS) using the reverse phase Radial Pak-A cartridge (8mm ID, μ-Bondapak C-18) and the Model RCM-100 module. A precolumn packed with μ-Bondapak $C_{18}$/Corasil preceded the system.



## REAGENTS AND STANDARDS

All solvents were glass-distilled (Waters Associates, Milford, MA) and filtered before use through a 0.45 μ Millipore filter (Millipore Corporation, Bedford, MA).

Water was obtained after deionization and reverse osmosis using a Culligan water system.

Phosphate buffer - pH 6.0: Dissolve 71 gm of anhydrous $Na_2HPO_4$ (Mallinckrodt Inc., Paris, KY) in 500 ml of water. Adjust to pH 6.0 with 12N HCL. Stable one year at room temperature.

(For Group I Drugs) Extraction solvent with Internal Standard (5-(p-methylphenyl -5- phenylhydantoin): Add 5.0 mg of 5-(p-methylphenyl)-5-phenylhydantoin (Aldrich Chemical Co., Milwaukee, WS) to 42 ml of absolute ethanol and dilute to 500 ml with chloroform. Stable six months in dark bottle at room temperature.

(For Group II Drugs) Extraction solvent with internal standard (hexobarbital): Add 2.0 mg of hexobarbital (Applied Science Labs, Inc., State College, PA) to 14 ml of absolute ethanol and dilute to 200 ml with chloroform. Stable six months in dark bottle at room temperature.



Anticonvulsant Stock Standard: Add 25.0 mg of phenobarbital (Applied Science Labs, Inc.) 25.0 mg of ethosuximide (Park Davis and Co., Detroit, MI), 15.0 mg of phenytoin (Applied Science Labs, Inc.), 15.0 mg of primidone (Applied Science Labs, Inc.) and 10.0 mg of carbamazepine (Ciba-Geigy, Summitt, NJ) to 3 ml of ethanol and dilute to 100 ml with methanol. Add 2-3 drops of chloroform and mix well to solubilize carbamazepine. Stable six months in dark bottle at 4°C.

Sedative Stock Standard I: Add 20.0 mg of methyprylon (Applied Science Labs, Inc.), 20.0 mg of butabarbital (Applied Science Labs, Inc.), 20.0 mg of butalbital (Ganes Chemicals, Pennsville, NJ), 20.0 mg of mephobarbital (Applied Science Labs, Inc.), 20.0 mg of glutethimide (Ganes Chemicals), and 20.0 mg of secobarbital (Applied Science Labs, Inc.), and dilute to 100 ml with methanol. Stable six months in dark bottle at 4°C.

Sedative Stock Standard II: Add 20.0 mg of phensuximide (Applied Science Labs, Inc.), 20.0 mg of amobarbital (Applied Science labs, Inc.), 20.0 mg of pentobarbital (Applied Science Labs, Inc.), and 20.0 mg of methaqualone (Applied Science Labs, Inc.), and dilute to 100 ml with methanol. Stable six months in brown bottle at 4°C.

Sedative Working Standard I: Add 1 ml of Anticonvulsant Stock Standard and 1 ml of Sedative Stock Standard I and dilute to 10 ml with drug-free serum. Aliquots are frozen and stable 6 months.

Sedative Working Standard II: Add 1 ml of Sedative Stock Standard II and dilute to 10 ml volume with drug-free serum. Aliquots are frozen and stable 6 months.

Mobile Phase: Mix 640 ml of 0.001 M $K_2HPO_4$ pH 4.4, 210 ml methanol, and 140 ml $CH_3CN$, filter and degas. Stable 2 days at room temperature.

Phosphate Buffer - 0.001 M, pH 4.4: Add 0.228 gm of $K_2HPO_4$ (Mallinckrodt, Inc., Paris, KY) to 1000 ml water. Adjust to pH 4.4 using 10% phosphoric acid (Mallinckrodt, Inc.). Stable 2 weeks at 4°C.

## METHODS

Procedure: If group I drugs (ethosuximide, primidone, methyprylon, phenobarbital, butabarbital, butalbital, phenytoin, glutethimide, carbamazepine, secobarbital, mephobarbital) are suspected pipette 100 µl of working standard I and 100 µl of patient serum into separate 3 ml glass centrifuge tubes. Add

100 µl of phosphate buffer, pH 6.0, to each tube. Add 0.4 ml of extraction solvent containing the internal standard (5-(p-methylphenyl)-5-phenylhydantoin).Vortex for 15 seconds and centrifuge for 5 minutes at 2000 rpm. Transfer the chloroform layer to a glass tube and evaporate to dryness at room temperature under nitrogen (10 cfh). Right before injection into the chromtograph add 50 µl of mobile phase to the tube and vortex. Inject at least 10 µl into the chromatograph. Injections may be made every 20 minutes or immediately after the 5-(p-methylphenyl)-5-phenylhydantoin peak elutes.



If group II drugs (phensuximide, amobarbital, pentobarbital or methaqualone) are suspected in the patient sample, working standard II and the extraction solvent containing the hexobarbital internal standard should be substituted for the working standard I and extraction solvent with 5-(p-methylphenyl)-5-phenylhydantoin internal standard in the above procedure.

Liquid chromatograph conditions are as follows:

Mobile Phase: 640 ml 0.001 M $K_2HPO_4$, pH 4.4, 210 ml MeOH, 140 ml $CH_3CN$
Flow Rate: 6.0 ml/min.
Back Pressure: 1800 psi
Wavelengths: 195 and 254 nm.
Sensitivity: 195-0.1 aufs, 254 - 0.005 aufs.
Chart Speed: 0.2 in/min.

## RESULTS AND DISCUSSION

The use of a buffered chloroform extraction instead of acetonitrile precipitation yields a cleaner sample with less

protein which extends column life. The addition of the buffer (pH 6.0) extracts phenobarbital with greater recoveries into the chloroform solvent (7). Ethosuximide was found to be very sensitive to heat and prolonged evaporation times. If ethosuximide is suspected the extraction solvent should be evaporated at room temperature and reconstituted with mobile phase immediately after evaporation is complete to assure good recoveries. The use of the RCSS resulted in sharp chromatographic peaks with a relatively fast analysis time of less than 20 minutes.



## Quantitation

Chromatographic peaks are identified using two techniques. Initially the identity of each drug is determined by its relative retention time as compared to the standards (see Table 1 and Figures 1 and 2). All drugs were quantitated with the ratio of peak height of the drug to the internal standard using 195 nm

TABLE 1
RETENTION TIMES (MIN)

| Drug | Time (Min) | Drug | Time (Min) |
|---|---|---|---|
| Ethosuximide | 1.4 | Phenytoin | 8.6 |
| Primidone | 1.8 | Amobarbital | 8.6 |
| Methyprylon | 2.7 | Pentobarbital | 8.6 |
| Phenobarbital | 3.2 | Glutethimide | 9.4 |
| Phensuximide | 3.4 | Carbamazepine | 10.8 |
| Butabarbital | 4.1 | Secobarbital | 12.4 |
| Butalbital | 5.1 | 5-(p-methylphenyl)-5-phenylhydantoin | 18.0 |
| Hexobarbital | 7.1 | | |
| Mephobarbital | 7.6 | Methaqualone | 19.6 |

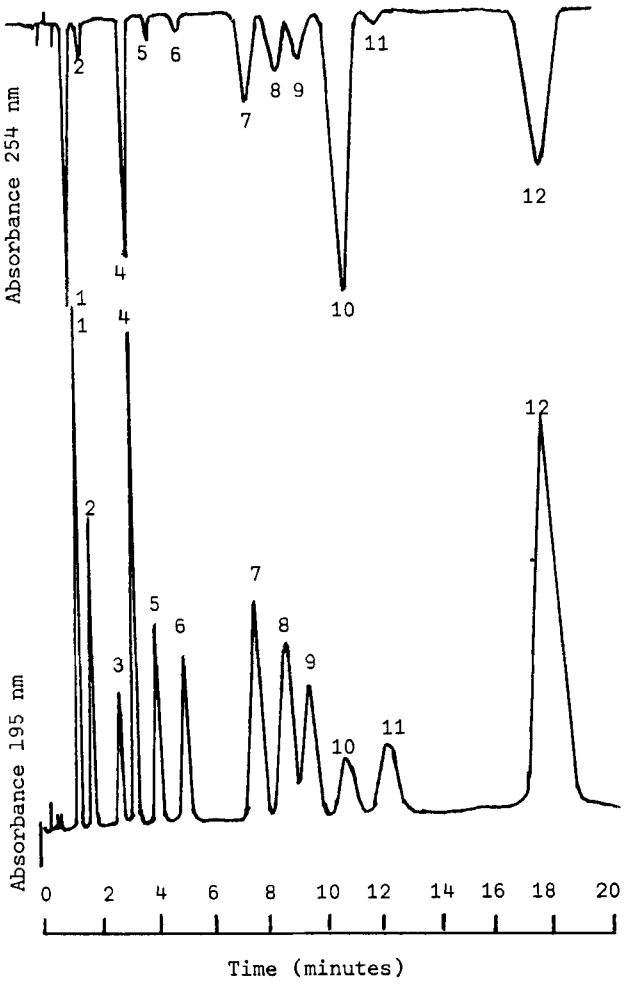


FIGURE 1

Chromatogram of group I drugs: 1. ethosuximide 2. primidone 3. methyprylon 4. phenobarbital 5. butabarbital 6. butalbital 7. mephobarbital 8. phenytoin 9. glutethimide 10. carbamazepine 11. secobarbital 12. 5-(p-methylphenyl)-5-phenylhydantoin (internal standard). Conditions: mobile phase, .001 M $K_2HPO_4$ (pH 4.4)/MeOH/$CH_3CN$ (64:21:14), 6.0 ml/min, 0.2 in/min, Waters Radial-pak A cartridge, RCSS.

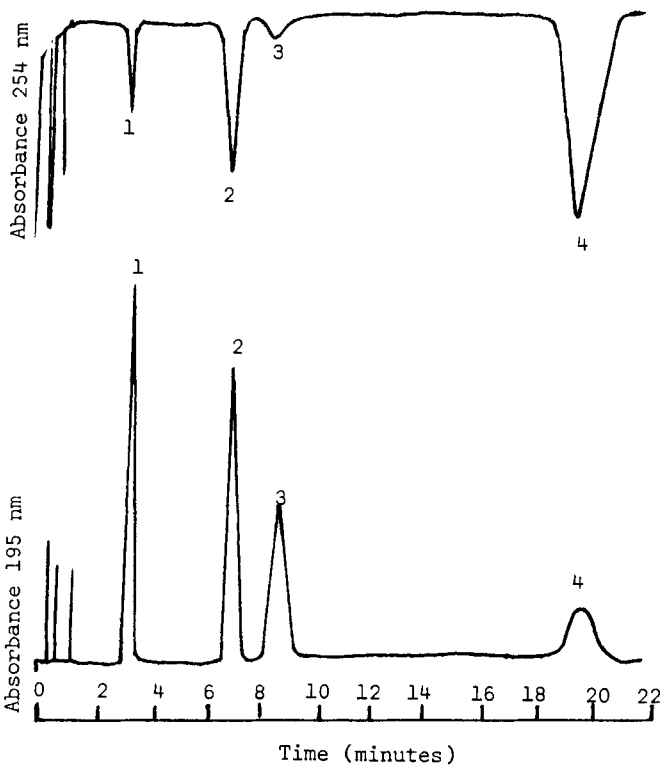


FIGURE 2

Chromatogram of group II drugs: 1. phensuximide 2. hexobarbital (internal standard) 3. amobarbital and pentobarbital 4. methaqualone. Conditions: mobile phase, .001 M $K_2HPO_4$ (pH 4.4)/MeOH/$CH_3CN$ (64:21:14), 6.0 ml/min, 0.2 in/min, Waters radial-pak A cartridge, RCSS.

except 254 nm was used for carbamazepine and methaqualone due to their low absorbance at 195 nm.

In addition to the retention time, an "R value"was determined for each drug to help confirm its identity. The "R value" was determined by utilizing the characteristic absorbance of each drug at the two different wavelengths (195 nm, 254 nm) and dividing

TABLE 2
R VALUE OF DRUGS

| Drug | A195/A254 (± 2SD) |
|---|---|
| Ethosuximide | 2.95 (± 0.33) |
| Primidone | 3.33 (± 0.81) |
| Methyprylon | low abs 254 |
| Phenobarbital | 0.95 (± 0.05) |
| Butabarbital | 3.02 (± 0.54) |
| Butalbital | 3.27 (± 1.08) |
| Mephobarbital | 0.94 (± 0.08) |
| Phenytoin | 1.31 (± 0.28) |
| Glutethimide | 1.19 (± 0.24) |
| Carbamazepine | 0.10 (± 0.01) |
| Secobarbital | 2.80 (± 1.39) |
| Phensuximide | 2.41 (± 0.17) |
| Methaqualone | 0.12 (± 0.02) |
| Amobarbital | 4.35 (± 1.37) |
| Pentobarbital | 4.35 (± 1.37) |



the absorbance of the drug to the internal standard at 195 nm by the absorbance of the drug to the internal standard at 254 nm:

$$R = \frac{\dfrac{\text{Peak Height Drug (at 195 nm)}}{\text{Peak Height Internal Standard}}}{\dfrac{\text{Peak Height Drug (at 254 nm)}}{\text{Peak Height Internal Standard}}}$$

R values were calculated for at least 20 samples of each drug at different concentrations and a range ($\pm$ 2 S.D.) was established, (see Table 2). An R value could not be established for methyprylon because of its low absorbance at 254 nm. Pheny-

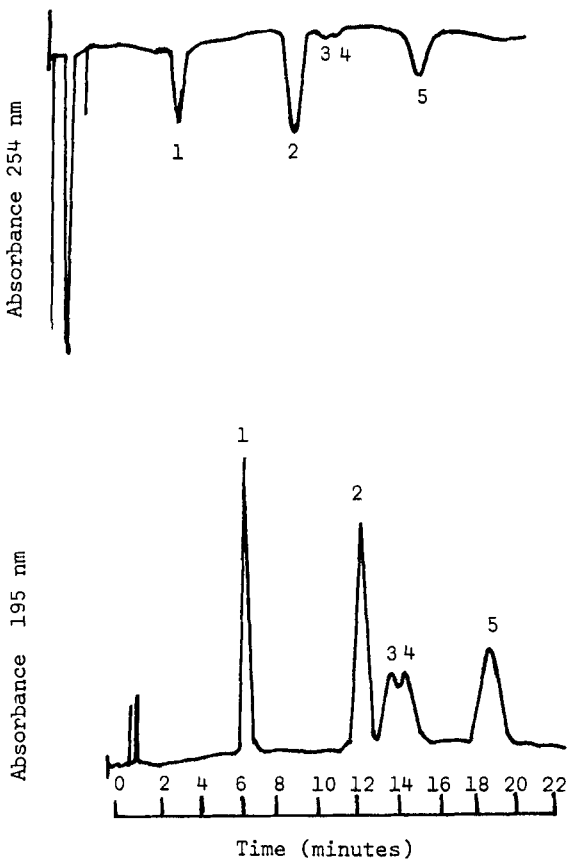


FIGURE 3

Chromatogram showing separation of phenytoin from amobarbital and pentobarbital using mobile phase of 80% .001 M $K_2HPO_4$ (pH 4.4) and 20% $CH_3CN$. 1. phensuximide 2. hexobarbital (internal standard) 3. pentobarbital 4. amobarbital 5. phenytoin.

toin, amobarbital and pentobarbital all have identical retention times. Phenytoin can be easily identified from amobarbital and pentobarbital by its greater absorbance at 254 nm and thus lower "R value". These drugs also can be separated by using the following mobile phase: 80% 0.001 M $K_2HPO_4$ (pH 4.4) and 20% $CH_3CN$. Figure 3 illustrates a typical chromatogram using this mobile phase.

Thus far in our experience we have not seen any interfering drugs with similar retention times and "R values" as the drugs of interest.



## Sensitivity

All the drugs could be detected and quantitated at a concentration of 3 µg/ml of serum. Sensitivity can be increased five-fold by injecting up to 50 µl of the extract.

## Linearity

Ethosuximide, methyprylon, phenobarbital, butabarbital, butalbital, mephobarbital, glutethimide, secobarbital, phensuximide and methaqualone were all found to be linear from 3 µg/ml to 100 µg/ml. Primidone and phenytoin are linear from 2 µg/ml to 75 µg/ml and carbamazepine 2 µg/ml to 50 µg/ml.

## Precision

Day-to-day precision was evaluated over a one month period by processing drug free serum containing each of the drugs in the concentrations shown in Table 3.

TABLE 3
DAY TO DAY PRECISION (N=23)

| Drug | Mean (µg/ml) | SD | CV (%) |
|---|---|---|---|
| Butabarbital | 25.0 | 2.0 | 8.1 |
| Butalbital | 24.9 | 2.0 | 7.9 |
| Carbamazepine | 8.2 | 0.6 | 7.6 |
| Ethosuximide | 98.3 | 4.4 | 4.5 |
| Glutethimide | 25.2 | 1.9 | 7.5 |
| Mephobarbital | 24.8 | 2.0 | 8.0 |
| Methaqualone | 25.3 | 1.6 | 6.5 |
| Methyprylon | 25.3 | 2.6 | 10.4 |
| Phenobarbital | 41.9 | 2.7 | 6.4 |
| Phensuximide | 25.3 | 1.4 | 5.6 |
| Phenytoin | 18.8 | 1.0 | 5.2 |
| Primidone | 12.1 | 1.1 | 9.1 |
| Secobarbital | 24.6 | 1.8 | 7.3 |



## CONCLUSION

This HPLC procedure has been used in our toxicology department as an initial screening test to detect the presence of sedative, hypnotic and anticonvulsant drugs. If any of these drugs are detected immediate quanitation can be performed and reported. In addition to emergency toxicology, the procedure is sufficiently specific and sensitive to be used for therapeutic drug monitoring. The use of the Radial Compression Separation System results in rapid analysis time, increased resolution, and faster equilibrium when changing mobile phases.

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