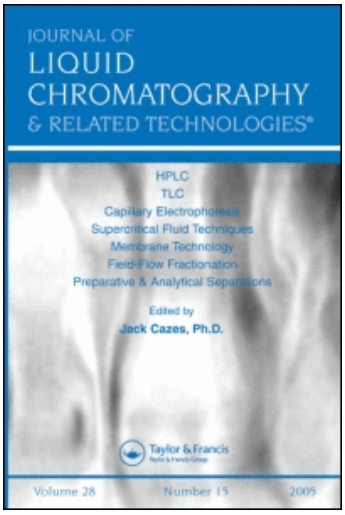

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The Identification of Drugs of Abuse in Urine Using Reverse Phase High Pressure Liquid Chromatography

Donald N. Harbin[a]; Peter F. Lott[a]
[a] University of Missouri-Kansas City, Kansas City, Missouri

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THE IDENTIFICATION OF DRUGS OF ABUSE IN URINE USING REVERSE PHASE HIGH PRESSURE LIQUID CHROMATOGRAPHY

Donald N. Harbin and Peter F. Lott
University of Missouri-Kansas City
Kansas City, Missouri 64110

ABSTRACT

A rapid, isocratic HPLC procedure for the identification of drugs of abuse in urine is described. The procedure utilizes a reverse phase μC_{18} column, a methanol/water mobile phase, buffered to pH 7.5, and a UV detector operating at 254 nm. Using indole as an external standard, 13 common drugs of abuse can be differentiated in less than 15 minutes. Nine different drugs of abuse were identified in actual drug screen urine samples, confirming TLC results. Reproducibility and quantitative capabilities of this method were also demonstrated. Due to apparent interferences from drug metabolites this method must at present be used in conjunction with another established method such as TLC or GC for positive drug identification.

INTRODUCTION

The most commonly used methods of detecting drugs of abuse in biological fluids employ thin layer chromatography (TLC) and/or gas chromatography (GC). Although these methods often give satisfactory results there still remain situations in which the ambiguities of a TLC plate or the necessity of making a volatile derivative compound for GC analysis makes these techniques less than ideal. High pressure liquid chromatography (HPLC) is a technique that holds much

promise for improving drug analyses. Because of the relative newness of the technique, a limited amount of work has been done in utilizing HPLC for toxicological analysis.

Christie, et al. (1), developed an HPLC method to detect LSD in urine using fluorescence detection and Jane and Taylor (2) used fluorescence to detect morphine in urine. Bugge (3) devised an HPLC method to detect diazepam in blood using UV detection; Dixon and Stoll (4) also employed a UV detector in their HPLC method to analyze blood serum for barbiturates. However, no work has been done previously to devise a simple, rapid, isocratic HPLC method for the identification of common drugs of abuse in urine samples.

EXPERIMENTAL

Apparatus

A Waters Associates (Milford, MA) model ALC 202 liquid chromatograph with a U6K injector, 254 nm fixed wavelength UV detector and a μ-Bondapak C_{18} reverse phase column was employed for all analyses.

Reagents and Samples

All solvents and buffer components were purchased from the Fisher Scientific Co. (Pittsburgh, PA). Drugs were purchased in the form of the prescription pharmaceutical products.

The solvents composing the mobile phase were filtered and degassed before use. Methanol was filtered through a 0.5 μm fluorocarbon membrane filter, while the aqueous buffer solution was filtered through a 1.2 μm cellulose ester membrane filter.

Drug-containing urine samples were obtained from the toxicology laboratories of the Children's Mercy Hospital, Baptist Memorial Hospital, and Upsher Laboratories, all of Kansas City, Missouri. The urine samples were known to contain a drug of abuse based either on the results of conventional TLC methods or the

fact that the drug had been administered to a patient as part of the hospital treatment.

Mobile Phase

The mobile phase, buffered to pH 7.5, consisted of a methanol/buffered water solution (60:40). The aqueous component of the mobile phase, a pH 6.2 buffer, consists of 125 ml of 0.1 M KH_2PO_4 and 20.3 ml of 0.1 N NaOH diluted to 750 ml with water. The buffer solution was adjusted to pH 6.2 using 85% H_3PO_4 or 0.1N NaOH as required.

The mobile phase is pumped at a flow rate of 0.8 ml/minute and the recorder is set at 0.5 inch (12.7 mm)/minute. This chart speed resulted in relatively broad peaks but greatly aided in the drug identification process by reducing the relative error in the retention time measurements.

Procedure: Standard Solutions

A set of standard solutions for the drugs of abuse was prepared in the following manner. Tablets were ground into a fine powder using a mortar and pestle; contents of capsules and aqueous solutions were used without further preparation. The drug sample was dissolved in 10 ml of water in a 30 ml separatory funnel, followed by an addition of dilute HCl or NH_4OH/NH_4Cl pH 10 buffer to adjust the pH. For acidic and neutral drugs the solution was adjusted to pH 3. For basic drugs sufficient buffer was added to obtain a pH 10 solution. The free drugs were then extracted with a single 10 ml portion of chloroform/2-propanol (9:1). The organic layer was separated and evaporated in a hood to near dryness on a hot plate set at low heat. The residue was then redissolved in methanol. Drug identities were confirmed from chloroform extractions using IR spectroscopy.

Drug Screening Procedure

A 25 ml aliquot of the urine sample was placed in a 125 ml separatory funnel. One ml of 2N HCl was added, resulting in a solution of approximately pH 3. Any acidic or neutral drugs and their metabolites were then extracted from the urine with 20 ml of chloroform/2-propanol (9:1). After separating the organic layer, 2 ml of pH 10 buffer was added to the urine to adjust it to pH 10. Extraction with 20 ml of chloroform/2-propanol (9:1) isolated any basic drugs and metabolites contained in the urine. Samples that resulted in emulsification were centrifuged for 5 minutes. The pH 3 and pH 10 organic layers were then evaporated in a hood to a volume of about 1 ml using a hot plate set at low heat. The final 1 ml of each extract is evaporated using a stream of air to avoid considerable sample loss. The residue was then redissolved in 1 ml of methanol and filtered using a 0.5 um fluorocarbon membrane filter. A 5 µl portion of this extract was injected into the HPLC. The relative retention times of the resulting peaks were then compared to the relative retention times of the drug standards. Indole was used as an external reference standard in this drug screening procedure.

RESULTS AND DISCUSSION

Tables 1-3 list the retention times, both absolute and relative, of the drug standards tested from each of the three major classes of drugs of abuse - acidic, neutral, and basic. All the drugs except amobarbital and pentobarbital have unique retention times and can be differentiated in less than 15 minutes. Problems of very similar retention times for different pairs of drugs are only apparent, and not real, in half the cases. For example, the relative retention times of diazepam (2.03) and methamphetamine (2.06) are so similar that it appears the difficulty in distinguishing between the two drugs would be substantial. In this drug screening procedure, however, diazepam should appear only in the

TABLE 1

Retention Times and Capacity Factors of Acidic Drugs

Drug	Retention Time(min)	Relative Ret. Time	k'
Amobarbital	6.45	1.05	0.86
Pentobarbital	6.45	1.05	0.86
Phenobarbital	4.91	0.80	0.42
Secobarbital	7.18	1.17	1.07
Indole	6.14	1.00	0.77

TABLE 2

Retention Times and Capacity Factors of Basic Drugs

Drug	Retention Time(min)	Relative Ret. Time	k'
Amphetamine	8.84	1.44	1.55
Codeine	13.20	2.15	2.81
Meperidine	5.65	0.92	0.63
Methamphetamine	12.64	2.06	2.65
Morphine	10.87	1.77	2.14
Indole	6.14	1.00	0.77

TABLE 3

Retention Times and Capacity Factors of Neutral Drugs

Drug	Retention Time(min)	Relative Ret. Time	k'
Caffeine	4.98	0.81	0.44
Chlordiazepoxide	10.25	1.67	1.96
Diazepam	12.46	2.03	2.60
Glutethimide	6.39	1.04	0.85
Methaqualone	8.48	1.38	1.45
Indole	6.14	1.00	0.77

acidic urine extract chromatogram, while any methamphetamine should only appear in the basic urine extract chromatogram. A similar situation exists in the case of methaqualone and amphetamine. In two other instances (glutethimide (1.04) and amobarbital/pentobarbital (1.05), and phenobarbital (0.80) and caffeine (0.81)) both members of the drug pair would appear in the acidic urine extract chromatogram, thus making exact identification difficult in those cases by the HPLC procedure.

Reproducibility of retention times, a factor which is crucial in making correct identifications, is excellent on a day to day basis. Using a recorder chart speed of 12.7 mm (0.5 inch)/minute the standard deviation of the absolute retention time for 12 injections of diazepam-spiked urine extract was 0.6 mm. Similarly for secobarbital the value was found to be 0.9 mm.

As one would expect, the detection sensitivities of the drugs varied considerably, being a function of the molar absorptivities

TABLE 4

Drugs of Abuse Detected in Urine (Mobile Phase: Methanol / Water 60:40)

Drug	Number of Urine Samples	Average Observed Relative Ret. Time[a]	Average Deviation of Observed Rel. Ret. Time(%)[b]	Rel. Ret. Times for Other Observed Peaks
Pentobarbital[c]	1	1.05	0.0	0.83
Phenobarbital	9	0.77	2.1	1.54
Secobarbital	1	1.14	2.6	0.78,1.05
Amphetamine	4	1.43	1.1	1.03,1.19, 1.85,1.99, 2.09,2.31
Meperidine	1	0.96	4.4	0.71,1.01 1.42,1.64
Chlordiazepoxide	5	1.64	1.7	1.04,3,23 3.36
Diazepam	2	2.14	5.4	1.03,1.19
Glutethimide	1	0.97	6.7	1.44
Methaqualone	3	1.39	2.4	1.05,1.16 1.59

[a]Relative to indole

[b]Compared to the relative retention time of the drug standard

[c]Gastric fluid sample

of the compounds at 254 nm. As a class, the neutral drugs had the best UV absorption, with amounts as low as 5 ng of chlordiazepoxide being detected. Approximately 0.1 µg of morphine could be detected,

whereas the remainder of the drugs studied could usually not be detected in amounts smaller than 1 µg.

Table 4 lists the different drugs of abuse that have been detected in urine samples using this drug screening procedure.

Figures 1 and 2 are chromatograms of the pH 3 and pH 10 extracts, respectively, of blank urine. As can be seen, neither chromatogram contains significant peaks which would interfere with the drug screening process. Figures 3-5 are chromatograms of some of the identified drugs detected in the urine extracts. It could be assumed that the major, unidentified peaks in these chromatograms are due to drug metabolites, but this has not been confirmed.

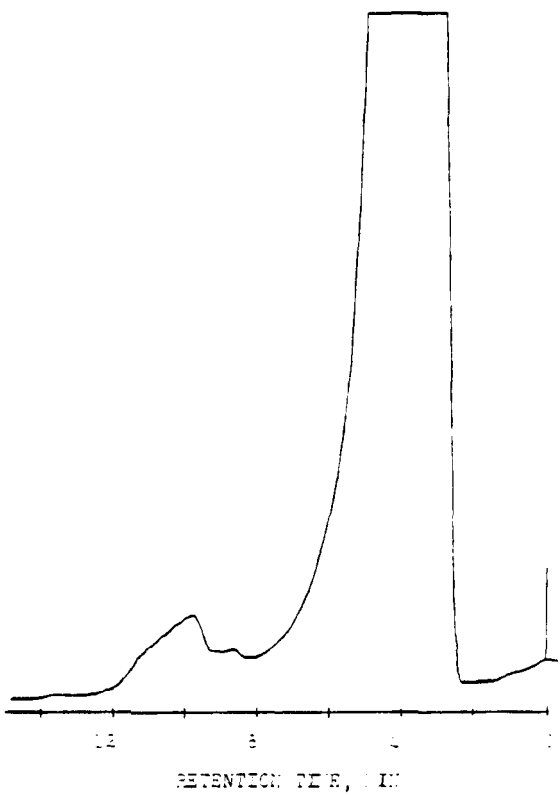

FIGURE 1

Blank urine chromatogram (pH 3 extraction).

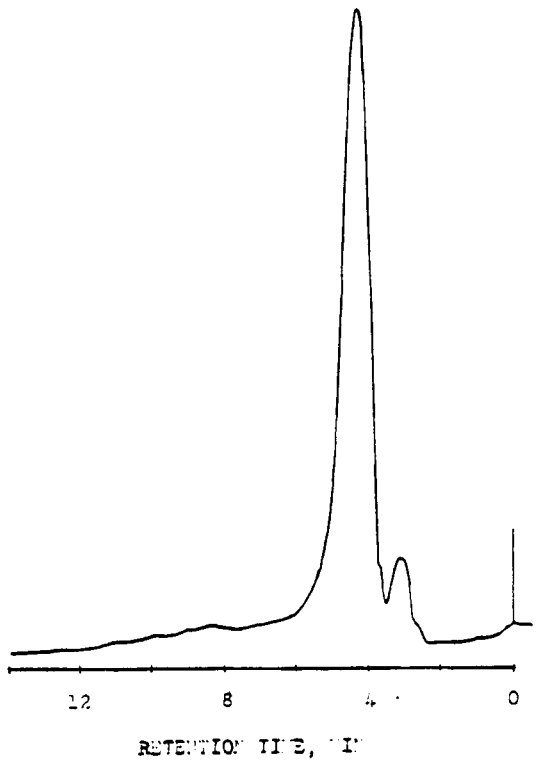

FIGURE 2

Blank urine chromatogram (pH 10 extraction).

The quantitative capability of this drug screen procedure was also investigated for several drugs. It was experimentally determined that a single chloroform/2-propanol extraction gave sufficient drug recovery for the majority of the drugs studied. Since the calibration curves necessary for quantitation are derived using the same extraction procedure, the extraction efficiency does not have to be maximized. Figure 6 shows the calibration curve that was determined for methaqualone. The curve does not pass through zero on the graph because, using spiked urines, the methaqualone elutes on the tail of the peak that results from normal urine constituents (see Figure 1). Using this method the concentration of methaqualone in the urine sample represented by

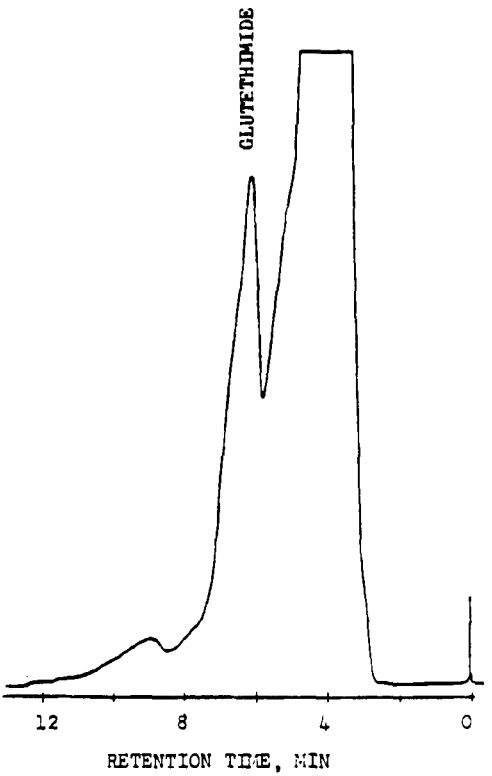

FIGURE 3

Glutethimide urine chromatogram.

Figure 4 is estimated to be 0.9 µg/ml. Apparently, not all drugs can be successfully quantitated using this drug screen procedure. Efforts to determine the calibration curve for phenobarbital proved unsuccessful, evidently as a result of interference from the large peak due to normal constituents in the urine, which elutes just prior to the phenobarbital. Several urine samples known to contain morphine were tested and in no case was morphine ever observed. It is concluded that the levels of free morphine in urine samples are insufficient to be detected using this method, the majority of morphine being excreted in water soluble glucuronide complexes and hence not extractable from the urine. This conclusion is predicated on the assumption that the morphine con-

taining urines which were tested were "typical" (a fact which cannot conclusively be determined, however).

The effect, if any, that drug metabolites would have on drug identification was uncertain at the beginning of this work. As the work progressed, it became apparent that metabolites are a very important factor in this drug screen procedure. This is due to the fact that the metabolites of some drugs have the same or similar retention times of other drugs. This means that the possibility of false drug identifications is significant if the identification is based on the retention time of the parent drug alone. A positive drug identification can only be made if one or more drug

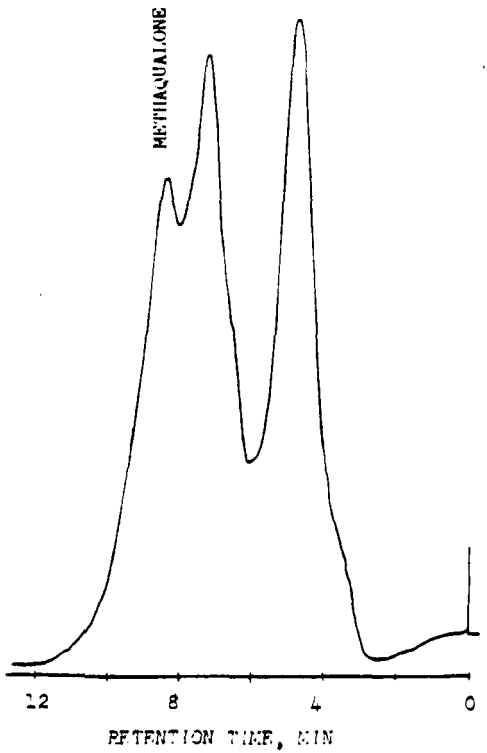

FIGURE 4

Methaqualone urine chromatogram.

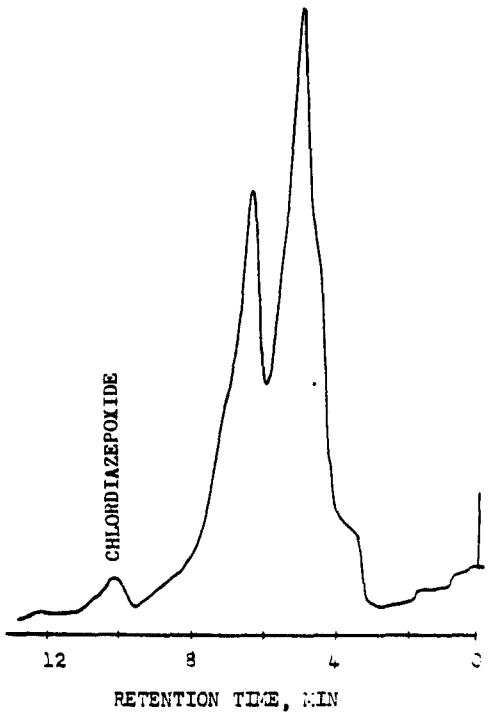

FIGURE 5

Chlordiazepoxide urine chromatogram.

metabolites as well as the original drug can be identified or if a second method of identification, such as GC or TLC, is employed to confirm the results. The first alternative is the most desirable, since it would require no additional analyses. At the present time, however, the retention times of the major metabolites of drugs of abuse are not known under the experimental conditions of this drug screening procedure. Therefore, this HPLC procedure at present must be used in conjunction with other drug identification methods. Once the retention times of the major drug metabolites are cataloged this HPLC procedure could be used exclusively, saving much analysis time and eliminating ambiguities which plague many present methods of analysis.

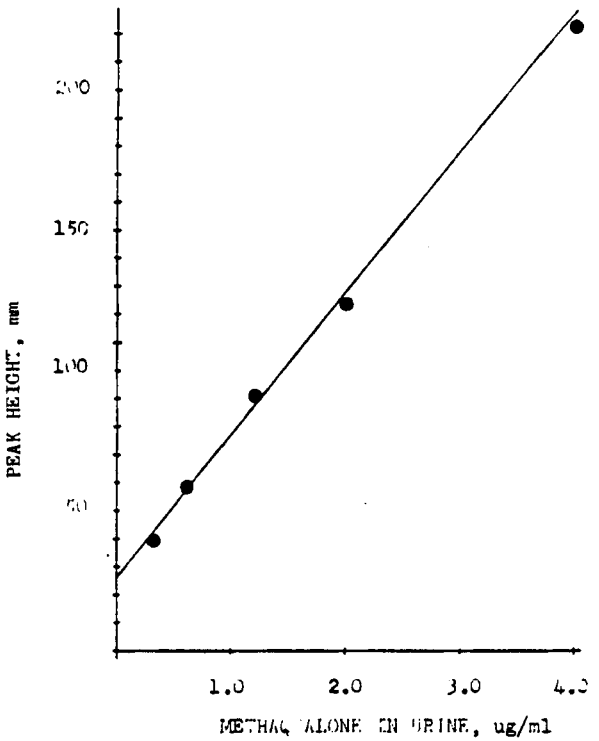

FIGURE 6

Calibration curve for methaqualone in urine.

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