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AUTOMATED LIQUID CHROMATOGRAPHIC ANALYSIS OF DRUGS IN URINE BY ON-LINE SAMPLE CLEANUP AND ISOCRATIC MULTI-COLUMN SEPARATION

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SUMMARY

A multi-column system has been developed for automated analysis of basic drugs in urine. Two polymeric pre-columns, containing PRP-1 and Aminex A-28, were used to isolate the drugs. A short reversed-phase column, coupled to a 150 × 4.6 mm I.D. silica column, produced the analytical separation. Sample preparation consisted of dilution and centrifugation. The entire procedure required less than 30 min. Careful optimization of mobile phase conditions led to retention of benzoylecgonine and barbiturates. For most drugs, levels of 0.3 mg/l were sufficient to produce peaks that could be matched against stored spectra with a computerized library search program.

INTRODUCTION

Analysis of drugs in biological fluids by liquid chromatography (LC) has been automated by a variety of techniques for on-line sample processing (OSP)[1,2]. The use of a short pre-column to separate the analytes from proteins and salts has been explored for over ten years[3,4]. Many current approaches and applications were recently reviewed[5,6]. Other useful approaches include zone electrophoretic sample treatment[7] and dialysis[8]. Furthermore, direct analysis of biological fluids is possible by the use of columns with hydrophilic surfaces[9,10] or of a micellar mobile phase that solubilizes proteins[11]. A device for the complete automation of sample handling for LC was recently described[12].

Many published applications of OSP have been restricted to a single drug and its metabolites. This undoubtedly reflects the widespread use of the technique for pharmacokinetic and bioavailability studies of new drugs. In some cases, drugs with similar chemical structures have been analyzed simultaneously. Examples include the analysis of benzodiazepines[13], amphetamines[14], barbiturates[15], and tricyclic antidepressants[16,17].

A number of difficulties have prevented the use of OSP for screening multiple classes of drugs in a single procedure. First, conditions for adsorption and desorption of drugs from a pre-column require careful optimization. Different strategies are appropriate for hydrophilic, moderately hydrophobic, and very hydrophobic drugs[18]. Second, techniques for broad-spectrum LC analysis of drugs have been reported in the

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literature[19,20] but have not been widely used. This reflects the success of gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS), which have gained more prominence for drug screening. Also, there is little qualitative information available from LC with detection at a single wavelength, as it is currently performed in most toxicological laboratories.

Recently, there have been several reports of LC screening techniques for toxicology where off-line sample preparation was used. Search programs were developed to analyze spectra collected with a diode-array detector. In order to determine acidic, neutral, and basic drugs in a single analysis, Demorest *et al.*[21] used an acetonitrile gradient at pH 2.1. Hill and Lagner[22] used two different gradients and two different columns for separate analyses of acids and bases. Isocratic methods for screening a limited group of hydrophobic drugs were reported by Minder *et al.*[23] and Jinno *et al.*[24].

In general, isocratic analysis would be preferable for full-scan UV detection[25]. The background contributed by the mobile phase would then not change during chromatography, and spectral libraries could be collected without reference to background spectra at a given retention time. This is particularly important at trace levels. However, isocratic reversed-phase chromatographic retention of multiple drug classes is difficult to achieve. In particular, amphetamines and opiates are very hydrophilic and are not readily analyzed under the conditions appropriate for benzodiazepines and tricyclic antidepressants.

Cation-exchange chromatography permits isocratic analysis of drugs under conditions where hydrophobic interactions are minimized. Jane[26] demonstated the use of underivatized silica, a weak cation exchanger, for drug analysis in 1975. Three different types of eluents have been reported: methanol containing ammonia[26–28], a completely aqueous mobile phase at a low pH[29], or mixtures of aqueous buffers with organic solvents[30–33]. In the latter two cases, a basic amine modifier was added to improve peak symmetry. The published methods are best suited for the analysis of strongly basic drugs; weak bases, like the benzodiazepines, show little retention on silica, even at basic pH[26]. Comparisons of the performance of underivatized silica to hydrophobic bonded silica have been published[34,35], and the optimization of mobile phase components has been investigated[16,35–37]. The interlaboratory reproducibility of retention times on underivatized silica has been reported[38].

Coupled-column chromatography is a useful isocratic alternative to solvent gradients, especially when repetitive analysis is required[39]. The versatility of underivatized silica may be extended by combination with a column displaying a different selectivity. Complex pharmaceutical mixtures have been analyzed by using column switching techniques and combinations of silica and reversed-phase packings[40]. Other investigators have coupled cation-exchange and reversed-phase columns. The combination of a 250 × 4.6 mm I.D. strong-cation-exchange column, coupled with a 50 × 4.6 mm I.D. reversed-phase column for drug analysis has been reported[41]. Illicit heroin samples were analyzed with a 45 × 2.1 mm I.D. C_{18} column, coupled with a 250 × 4.6 mm I.D. alumina column[42].

We have developed a multi-column system for the analysis of drugs in urine. Primary sample clean-up is performed with a 10 × 2.1 mm I.D. PRP-1 cartridge. A 10 × 3.2 mm I.D. anion-exchange column is used to selectively retain hydrophobic neutral and acidic drugs. Under carefully optimized conditions, barbiturates are

slightly retained by the anion-exchange column. Finally, coupled reversed-phase (25 × 3.2 mm I.D.) and silica (150 × 4.6 mm I.D.) columns permit the separation of barbiturates, benzodiazepines, amphetamines, tricyclic antidepressants, and opiates. Column switching is employed so that only a small eluate volume from the first two columns reaches the final two columns. A diode-array detector is utilized for monitoring and identification of the eluted peaks.

EXPERIMENTAL

Apparatus

The apparatus was constructed from two high-pressure pumps, three high-pressure switching valves, one solvent selection valve, two pre-columns, two analytical columns, a heater, an automatic sampler, a UV detector and a system controller, as shown in Fig. 1.

A Bio-Rad (Richmond, CA, U.S.A.) Model 402 gradient controller with an ACER 710 personal computer controlled the functions of pump A (Model 1330 isocratic pump, Bio-Rad), the 8-port high-pressure valve (Valco, Houston, TX, U.S.A.) and two 4-port high-pressure valves (Valco). The controller initiated sampling via a signal to the Model AS-48 automatic sampler (Bio-Rad), equipped with a 500-μl sample loop. When the filled injection loop was rotated into the flow path, the

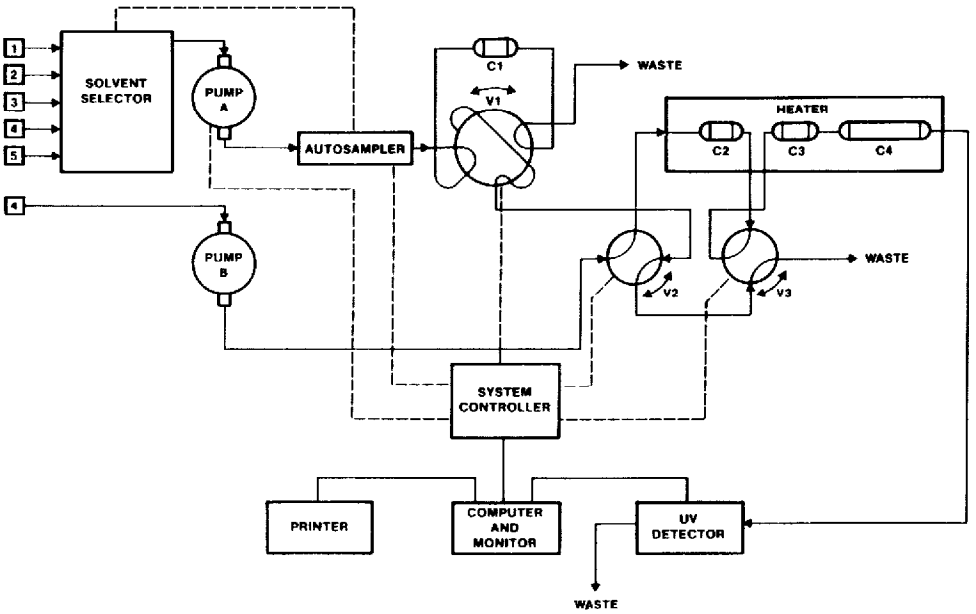

Fig. 1. Experimental set-up for on-line analysis of basic drugs in urine according to the final procedure described in the text. V1 = 8-port switching valve; V2, V3 = 4-port switching valves; C1 = 10 × 2.1 mm I.D. PRP-1 column, 16 μm; C2 = 10 × 3.2 mm I.D. Aminex A-28 column, 11 μm; C3 = 25 × 3.2 mm I.D. C_8 column, 5 μm; C4 = 150 × 4.6 mm I.D. silica column, 5 μm; 1 = 0.1% potassium borate buffer (pH 8.0) (buffer A); 2 = 6 m*M* KH_2PO_4, 5 m*M* tetramethylammonium hydroxide, 2 m*M* dimethyloctylamine, adjusted to pH 6.50 with phosphoric acid (buffer B); 3 = 60% buffer B, 40% acetonitrile; 4 = 67% buffer B, 33% acetonitrile; 5 = 30% buffer B, 70% acetonitrile.

automatic sampler signalled back to the controller and at the same time sent a signal to the solvent selector (FIATRON, Oconomonoc, WI, U.S.A.). These signals initiated the valve sequence and solvent selection sequence. A second Model 1330 isocratic pump ran at a constant flow-rate, independent of the controller. The first column was operated at ambient temperature, the other three were maintained at 40°C in a column heater (Bio-Rad). The chromatograms were routinely monitored at 210 nm and 235 nm with a Model 1040A diode-array detector (Hewlett-Packard, Avondale, PA, U.S.A.), which included a Model 85B microprocessor. Chromatograms generated at 210 nm were stored by the system controller. A Model 3392A Integrator (Hewlett-Packard) monitored the signal at 235 nm and performed quantitation at either wavelength, as required. For identification of drug spectra, a commercially available toxicology program (Library Search/HP 1040A, Central Pathology Laboratory, Santa Rosa, CA, U.S.A.) was used. A Model E Microfuge (Beckman, Fullerton, CA, U.S.A.) was employed in sample preparation.

Stationary phases and columns

Initial urine purification and drug concentration was performed with a 10 × 2.1 mm I.D. stainless-steel pre-column, packed with PRP-1, a spherical 12–20 μm poly(styrene–divinylbenzene) co-polymer (Hamilton, Reno, NV, U.S.A.). Further purification was performed with a 10 × 3.2 mm I.D. stainless-steel pre-column, packed with Aminex A-28 11-μm resin (Bio-Rad). Both pre-column cartridges were slurry-packed at 5000 p.s.i. and were held in Brownlee cartridge holders (Rainin, Emerville, CA, U.S.A.). The 25 × 3.2 mm I.D. reversed-phase cartridge contained 5-μm octylsilica (Phenomenex, Rancho Palos Verde, CA, U.S.A.) and was slurry-packed at 5000 p.s.i. The 150 × 4.6 mm I.D. column contained 5-μm spherical silica, 50-Å pore size (Machery-Nagel, Duren, F.R.G.) and was slurry-packed at 7500 p.s.i. A small cartridge containing ACT-1, a C_{18}-derivatized PRP column (Interaction Chemicals, Mountain View, CA, U.S.A.) and a small reversed-phase cartridge (Bio-Rad) were used in comparison studies.

Chemicals

HPLC-grade acetonitrile was obtained from Alltech Assoc. (Los Altos, CA, U.S.A.); HPLC-grade potassium dihydrogenphosphate was from Fisher (Santa Clara, CA, U.S.A.); tetramethylammonium (TMA) chloride and hydroxide were obtained from Fluka (Ronkonkoma, NY, U.S.A.), and N,N-dimethyloctylamine was from Aldrich (Milwaukee, WI, U.S.A.). All other laboratory chemicals were of analytical grade. Water was purified using an in-house ion-exchange system and was equivalent to HPLC grade. Drugs and organic acids were obtained from Alltech (State College, PA, U.S.A.) or Sigma (St. Louis, MO, U.S.A.). Other drugs were gifts from their respective manufacturers.

N-Ethylnordiazepam (7-chloro-1,3-dihydro-1-ethyl-5-phenyl-2H-1, 4-benzodiazepin-2-one) was synthesized in-house by N-ethylation of nordiazepam with ethyl iodide. It was recrystallized from methanol and showed a single peak when analyzed by reversed-phase chromatography under conditions where it is fully resolved from nordiazepam[43].

RESULTS AND DISCUSSION

Initial urine purification

The simultaneous extraction of drugs with varying charges and polarities from urine requires a very hydrophobic packing. XAD-2 resin has been employed for this purpose in an open-column mode for many years[44]. PRP-1 is a highly cross-linked polymer of identical chemical composition. The utility of this packing for extraction of methaqualone in serum was reported by Hux *et al.*[45]. The versatility of PRP-1 is illustrated by published procedures which demonstrate the direct extraction of both barbiturates and amphetamines from urine[14,15].

Our investigations verified the retention on PRP-1 of basic, neutral, and weakly acidic drugs from urine, buffered to the pH range 7.5–9.5. In particular, hydrophilic drugs, such as benzoylecgonine and ethchlorvynol, were retained under these conditions. When reversed-phase cartridges were evaluated under identical conditions, these hydrophilic drugs were not retained, and no single pH could be determined where both barbiturates and amphetamines were extracted from urine. The breakthrough volumes of all drugs in pH 8.0 buffer was sufficiently large, so that the flow through the PRP-1 column could be reversed without any losses. This permitted rinsing of the front end of the column, where particulates accumulate. The final wash volume was determined by the length of time required to rinse weakly retained urine components from the column, as determined by direct observation of the baseline at 210 nm.

The PRP-1 cartridge was very tolerant of biological specimens under the conditions employed. Fouling of the column by urine samples (which had been previously centrifuged at 11 000 *g*) was never observed. Experiments performed with pre-buffered and centrifuged serum indicated that at least 50 ml of serum could be injected before a substantial increase in backpressure occurred.

Elution from the first pre-column

The elution of drugs from the PRP-1 pre-column was attempted, using three different approaches: pH changes, addition of competing ions, or addition of organic solvents. Combinations of these methods were also evaluated.

A change in pH alone was sufficient to elute amphetamines and benzoylecgonine. Benzodiazepines and tricyclic antidepressants were not eluted, even at a very low pH, unless an organic modifier was added. However, low pH was not compatible with the use of a silica column as a cation-exchanger. Small amounts of the acid would inevitably reach that column, causing a reduction in the number of ionized silanol sites and a dramatic loss of retention. Elution at pH 6.5 required a higher concentration of acetonitrile than elution under acidic conditions.

Although amphetamines and other hydrophilic drugs were eluted from the PRP-1 cartridge in volumes below 100 μl, the hydrophobic drugs were eluted much more slowly; elution volumes for amitriptyline and diazepam were closer to 500 μl. Furthermore, elution of these drugs typically began after the hydrophilic drugs had been released. Since it was unlikely that the compounds had penetrated the column in the forward-flow mode, it would appear that the interaction with the packing was particularly strong. Experiments with reversed-phase cartridges of similar dimensions yielded peak widths of the eluted drugs that were only half as large. Cartridges packed

with ACT-1, a poly(styrene–divinylbenzene) co-polymer which contains covalently bonded octadecyl groups, yielded 2- to 3-fold increases in the elution volumes for diazepam and amitriptyline. PRP-1 cartridges of different length and inner diameter yielded nearly identical elution volumes.

Addition of basic competing agents to increase the efficiency of elution for hydrophobic drugs was also evaluated. Dimethyloctylamine concentrations (0.005 *M*) and tetramethylammonium chloride (0.4 *M*) led to no significant changes in the width of eluted peaks.

Because the two analytical columns contain silica and would be adversely affected by basic buffers, the preliminary replacement of borate buffer with pH 6.5 phosphate buffer was studied. The dilute borate buffer was displaced with 250 μl of 6 m*M* buffer (containing the competing bases). Larger volumes led to the elution of amphetamines. This buffer replacement step had a significant effect on the lifetime of the silica columns. In the absence of any wash step, a large void would sometimes form at the front end of the silica column after less than 50 injections. With the displacement step, this column could be used for over 200 injections. The buffer replacement step also had a favorable effect on the peak shapes of the hydrophobic drugs. These compounds were concentrated at the head of the subsequent column. A stepwise-gradient was produced by solvent switching; drugs were transferred from the rear of the PRP-1 column to the front of the anion-exchange column at the reduced acetonitrile concentration and were briefly retained there.

Further purification of the urine extract on an anion-exchange column

Although most carboxylic acids are completely ionized at pH 8.0, many organic acids were retained on the first precolumn after the initial clean-up. We previously studied the hydrophobicity of many endogeneous phenolic and indolic acids[4,6]. Under the conditions employed here for clean-up, hippuric acid was not retained at all; indolepropionic acid was retained in the initial (forward) wash but was slowly washed off the column in the reverse wash. Acidic drugs, such as ibuprofen and indomethacin, were fully retained by PRP-1.

The use of a second pre-column, containing anion-exchange resin was therefore investigated. A polymeric material was most appropriate because of its high capacity and pH stability. Initial experiments with pre-packaged 30 × 4.6 mm I.D. Aminex A-27 cartridges (Bio-Rad) demonstrated extensive retention of endogenous and exogenous carboxylic acids. (Benzoylecgonine contains a carboxyl group, but is amphoteric; at pH 6.5 it displays weakly basic behavior.) However, barbiturates were also retained, along with oxazepam and several other benzodiazepines. To minimize this retention, smaller columns (10 × 3.2 mm I.D.) were prepared; also, a smaller particle size was employed (Aminex A-28; 11 ± 2 μm) to improve the peak shape of the weakly retained drugs. Attempts to pack narrower columns were not successful due to swelling of the resin in the presence of organic solvents.

When barbiturates were eluted from the A-28 column alone under isocratic conditions, the retention times were shorter than those observed following transfer from the PRP-1 column. This is due to the gradient which is generated on the A-28 column (Table I). In the absence of solvent, none of the acids are eluted when buffers with low ionic strength are employed. The addition of organic solvents helps to elute the barbiturates, but at intermediate solvent concentrations (*e.g.* 20%) phenobarbital

TABLE I

RETENTION DATA (k') FOR SELECTED COMPOUNDS ON A 10 × 3.2 mm I.D. AMINEX A-28 COLUMN

Eluent A = 6 m*M* KH_2PO_4, 5 m*M* tetramethylammonium chloride, 2 m*M* dimethyloctylamine (pH 6.50); eluent B = acetonitrile. Flow-rate, 1.0 ml/min; 40°C. The total void volume was 0.18 ml, as measured by injection of 50% aqueous methanol. φ is the phase ratio (B/A + B).

Compound	φ						
	0.1	*0.2*	*0.3*	*0.4*	*0.5*	*0.6*	*0.7*
Indolepropionic acid	>50	>50	>50	>50	39.1	26.6	17.9
Indomethacin	>50	>50	>50	41.5	31.6	20.7	11.0
Ibuprofen	>50	>50	>50	35.0	29.4	13.1	6.8
Phenobarbital	22.4	10.0	5.0	2.7	0.9	1.0	1.0
Secobarbital	10.5	4.6	1.8	0.8	0.5	0.4	0.3
Oxazepam	25.5	6.8	2.3	1.0	0.8	0.5	0.2
Nordiazepam	35.7	7.7	2.0	0.9	0.7	0.4	0.3
Diazepam	20.2	4.2	1.2	0.5	0.6	0.2	0.3
Imipramine	0.9	0.6	0.1	0.1	0.1	0.1	0.1
Amphetamine	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Caffeine	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Morphine	0.1	0.1	0.1	0.1	0.1	0.1	0.1

(pK_a 7.2) is released more slowly than secobarbital (pK_a 7.9). Furthermore, TMA in the mobile phase competed with the resin under conditions where partition occurred; an increase in the TMA concentration speeded elution of barbiturates. It was important to optimize anion-exchange conditions in order to minimize the contribution of endogenous acids to the background absorbance at 210 nm in the final chromatograms.

Coupled analytical columns

Silica columns have been used for drug screening in both basic and neutral solutions. Extended column stability at high pH was probably due to the very low concentration (less than 10%) of water in the mobile phase. Under conditions where the mobile phase would be useful for coupled-column chromatography with reversed-phase columns, the water content would be greater than 50%. Therefore, neutral eluents are more suitable for coupled-column analysis.

Mobile phase conditions appropriate for the separation of benzodiazepines on reversed-phase columns typically lead to lengthy retention of the more hydrophobic tricyclic antidepressants and phenothiazines. The addition of a competing base to the mobile phase[47] minimizes the difference in retention. This approach is successful because a significant proportion of retention is due to ionic interaction with the negatively charged silanol groups remaining on the bonded surface.

We recently demonstrated the use of N,N-dimethyloctylamine to reduce effectively the retention of tricyclic antidepressants on a reversed-phase column[43]. Therapeutically important benzodiazepines and tricyclic antidepressants were analyzed in 30% acetonitrile (pH 6.4) on a 100 × 2.1 mm I.D. 3-μm octylsilica column. For the screening procedure described here, reduction of column dimensions to give

minimum acceptable resolution of benzodiazepines was desirable. Resolution of chlordiazepoxide and its metabolite, norchlordiazepoxide, and of diazepam and its metabolites, nordiazepam and oxazepam, was evaluated. Analysis of the second group was complicated by the slight retention of oxazepam and nordiazepam on the A-28 column. A 25 × 3.2 mm I.D. reversed-phase column and 33% acetonitrile yielded acceptable resolution ($0.5 < R < 1.0$). With longer columns amitriptyline and morphine or other opiates were unresolved.

Quaternary amines were reported to improve the peak shapes of basic drugs, chromatographed on silica[30,31]. Furthermore, the total analysis time is responsive to both the concentration of quaternary amines and the cations in the buffer. Alkylamines also lead to improved peak shapes on bare silica. In agreement with earlier observations[37], we found that tetramethyl- and tetraethylammonium salts were not appreciably adsorbed on silica, and both improved peak shapes when present in small amounts. Ammonium buffers also improved peak shapes and appeared to speed preferentially the elution of primary amines.

In Table II the elution order of the coupled column system is compared to results obtained for the silica column alone. Some results are also shown from the extensive toxicological survey reported by Jane *et al.*[28], who employed a 125 × 4.9 mm I.D. Spherisorb 5-SW column; the mobile phase was methanolic ammonium perchlorate (10 m*M*, pH 6.7). It may be seen that the retention time and the elution order for silica with 33% acetonitrile is quite similar to that in the earlier study in which 90% methanol was used. Apparently, cation exchange is the primary retention mechanism under both conditions. Addition of the short reversed-phase cartridge resulted in slightly increased retention of benzodiazepines and permitted increased differentiation within this class. The acetonitrile concentration chosen for the analytical separation (33%) was lower than the optimal concentration for elution from PRP-1 at pH 6.5 (40%). However, a small amount of the higher concentration could be tolerated by the reversed-phase column without measurable effect on the reproducibility of retention times; this volume was experimentally determined to be 0.2 ml. The combination of this step with the borate buffer replacement described above led to compression of hydrophobic drugs on the anion-exchange column and improved peak shapes in the final separation.

The silica column must offer sufficient retention to assure elution of amphetamines after benzodiazepines (which are substantially retained on the reversed-phase column). Retention on underivatized silica is proportional to the surface area of the packing[48,49]. A 50-Å 5-μm packing with a surface area of 450 m^2/g produced sufficient retention in a 150 × 4.6 mm I.D. column to achieve the desired selectivity.

The coupled analytical columns and the anion-exchange column were maintained at 40°C to increase the reproducibility of retention times. This also improved peak shapes and lowered the backpressure.

Regeneration of the pre-columns

The elution of the PRP-1 column in the back-flush mode led to the release of most hydrophobic drugs, *e.g.*, buclizine, butaclamol, and emetine. Cyclosporine was the only one of the compounds evaluated that was retained. Experiments with drug-free serum revealed a significant peak not seen in urine. It was eluted at

TABLE II

RETENTION DATA (k') FOR DRUGS IN SINGLE- AND MULTI-COLUMN SEPARATIONS

Compound	*Complete system*[a]	*Coupled analytical columns*[b]	*Silica alone*[c]	*Literature*[d]
Caffeine	0.7	0.5	0.6	0.2
Cotinine	0.9	0.8	0.7	0.2
Benzoylecgonine	1.0	0.6	0.9	0.9
Secobarbital	1.0	0.6	0.4	N.A.
Oxazepam	1.1	0.6	0.5	N.A.
Phenobarbital	1.1	0.5	0.4	N.A.
Nordiazepam	1.3	0.8	0.5	0.2
Diazepam	1.6	1.0	0.5	0.1
N-Ethylnordiazepam	2.1	1.3	0.5	N.A.
Phenylpropanolamine	2.2	2.2	2.3	0.9
Phentermine	2.4	2.8	2.9	0.6
Amphetamine	2.5	2.5	2.5	0.9
Phenmetrazine	2.7	2.7	3.0	1.7
Lidocaine	2.7	2.4	2.5	0.6
Ephedrine	2.7	2.8	2.4	1.0
Pentazocine	2.8	3.4	3.5	1.8
Methamphetamine	3.1	3.1	3.1	2.1
Desipramine	3.1	3.1	3.1	2.1
Nortriptyline	3.3	3.0	3.0	2.0
Diphenhydramine	3.4	3.2	3.5	3.3
Methadone	3.8	3.4	3.4	2.2
Methadone metabolite[e]	4.1	4.1	4.2	2.8
Imipramine	4.2	3.7	3.7	4.2
Flurazepam	4.2	3.8	4.0	1.3
Amitriptyline	4.3	3.6	3.4	3.3
Morphine	5.0	5.1	5.7	3.8
Codeine	5.7	5.6	6.4	4.8
Chlorpheniramine	5.9	5.7	6.4	3.9
Hydromorphone	7.0	6.9	7.6	7.9
Hydrocodone	8.0	7.8	9.1	7.1

[a] Analysis in fully automated four-column system. Void volume, 1.9 ml; "injection point", time when fastest drugs are eluted from column 1.

[b] Isocratic analysis on reversed-phase and silica columns only. Void volume, 1.7 ml.

[c] Isocratic analysis on silica column only. Void volume, 1.5 ml.

[d] Reported retention data[28]. Mobile phase, 10 mM ammonium perchlorate (pH 6.7) in 90% aqueous methanol; column, 125 × 4.9 mm I.D. Spherisorb 5 SW. N.A. = data not available.

[e] 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine.

acetonitrile concentrations above 26% and was attributed to lipids. The A-28 column, which has the same polymeric structure as PRP-1, is eluted in the forward direction. Hydrophobic anions are strongly retained on the anion-exchange column, as are hydrophobic neutral compounds, including the lipids. A 70% acetonitrile wash facilitated the elution of these compounds. Furosemide was not eluted, even with 70% acetonitrile, due to the low ionic strength of the eluent.

Sample application

Experiments were conducted to determine the amount of buffer required to

neutralize an acidic urine (pH 5.0). A 1:1 mixture of urine with 2% potassium borate (pH 8.0) was required. Higher borate concentrations could not be employed due to limited solubility. Ammonium acetate was then examined because of its high solubility in water and acetonitrile. A 1:1 mixture of urine with 2 *M* ammonium acetate (pH 8.0) offered adequate buffering and also better peak shapes than the borate buffer. A 5:1 mixture of urine with 6 *M* ammonium acetate (pH 8.0) gave equivalent buffering capacity and permitted the injection of a more concentrated sample.

Final procedure

A total of five different eluents are employed in the complete system. Based on their function, these may be described in the following manner: (1) application buffer, (2) pH exchange buffer, (3) strong eluting buffer, (4) mobile phase, (5) pre-column wash solvent. The final experiments were performed using the instrumentation and formulations shown in Fig. 1.

Urine from healthy laboratory employees was spiked with eight drugs from a variety of chemical classes and with two internal standards, N-ethylnordiazepam and chlorpheniramine. Caffeine was present in almost all urine and was eluted near the solvent front. Spiked sample (0.5 ml) was mixed with 0.1 ml 6 *M* ammonium acetate (adjusted to pH 8.0 with 2 *M* potassium hydroxide) and centrifuged 30 s at 11 000 *g*. The 1.5-ml microcentrifuge tube was placed in the autosampler; the sampling needle was raised a few mm above the bottom of the cup to prevent contact with precipitate. Following injection, the pump A flow-rate was raised to 5 ml/min for 0.6 min. The flow-rate was briefly lowered while valve 1 (V1) was reversed, then the PRP-1 column was backflushed with 8 ml of the application buffer. The flow-rate was lowered to 1.0 ml/min. The solvent selector was switched to buffer 2 (0.25 ml) so that buffer 2 reached the PRP-1 column after the new flow-rate was achieved. The time of elution for amphetamine and morphine was determined by direct detection in the eluate of the first column; V2 was switched 3 s before this time to permit transfer of drugs to the A-28 column. V3 was switched 6 s later to permit transfer of rapidly eluted drugs to the coupled analytical columns. The time of elution of the slowest-eluted drugs from the A-28 column (secobarbital and oxazepam) was determined by direct detection in the eluate from that column. At this time, V3 was switched to disconnect the clean-up columns from the analytical columns. During this time, the solvent selector was switched to buffer 3 (0.2 ml), speeding elution of drugs from the PRP-1 column, and then to buffer 4. The total volume transferred from the PRP-1 column was 1.6 ml.

Following transfer of all drugs to the coupled analytical columns, the flow was maintained by pump B at 1.0 ml/min until analysis was complete. This pump delivered mobile phase continuously at a constant flow-rate; its output was diverted to waste during the transfer steps. While the analytical separation was completed, the pre-columns were rinsed with 7 ml of wash reagent (70% acetonitrile in buffer). The PRP-1 column was rinsed first with 2 ml; then the PRP-1 column and A-28 column were rinsed together. Next, both clean-up columns were equilibrated with mobile phase. When analysis was completed, V2 was switched so that pump B delivered mobile phase to columns 2, 3, and 4; pump A was used to equilibrate the PRP-1 column in borate buffer, while the next sample was loaded.

Fig. 2 shows the performance of the final three columns, eluted isocratically, after injection of 50 µl of an aqueous mixture containing 2–5 mg/l of each drug. Figs.

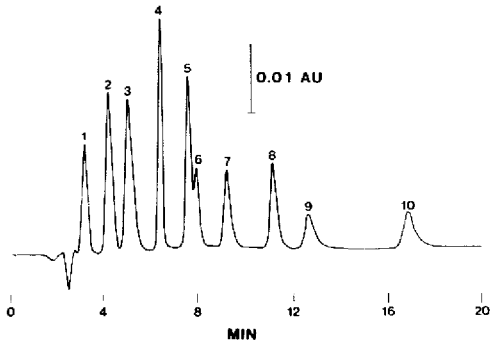

Fig. 2. Chromatogram produced by injection of 50 μl of an aqueous mixture into columns 2, 3, and 4. (Column 1 was replaced by a short length of 0.25 mm I.D. tubing.) Drugs shown are: (1) secobarbital (5 mg/l), (2) diazepam (2 mg/l), (3) N-ethylnordiazepam (4 mg/l), (4) amphetamine (5 mg/l), (5) methamphetamine (5 mg/l), (6) diphenhydramine (2 mg/l), (7) imipramine (2 mg/l), (8) morphine (2 mg/l), (9) chlorpheniramine (3 mg/l), (10) hydrocodone (2 mg/l). Mobile phase, 6 m*M* KH_2PO_4, 5 m*M* tetramethylammonium hydroxide, 2 m*M* dimethyloctylamine, pH 6.50 containing 33% acetonitrile; detection, 210 nm.

3 and 4 show the performance of the complete system for 500-μl injections of drug-free and drug-supplemented urine. It may be seen that preconcentration on PRP-1 resulted in noticeable broadening of the benzodiazepine and imipramine peaks; for other drugs the 500-μl injection caused very little broadening.

The system repeatability for ten consecutive injections is shown in Table III. Peak height and retention time precision were excellent for concentrations of 2–5 mg/l. Although little retention variability may be observed on a daily basis, changes may occur as the reversed-phase and silica columns age and retention decreases. The two internal standards may be useful to monitor these changes; N-ethylnordiazepam retention reflects the performance of the reversed-phase column, while chlorpheniramine retention reflects the performance of the silica column.

Linearity data for concentrations of 0.3, 1, 2, 5, 10, and 25 mg/l are shown in Table IV. Concentrations as high as 100 mg/l did not lead to carry-over in subsequent specimens.

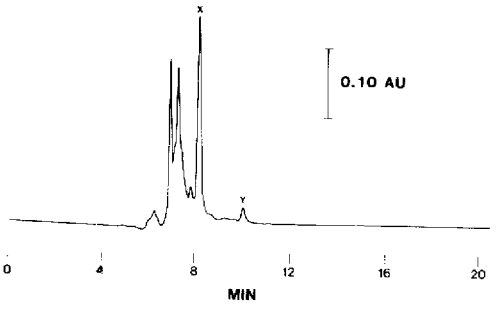

Fig. 3. Chromatogram of 500 μl of buffered drug-free urine, analyzed with the complete system. X and Y are endogenous peaks. Caffeine is eluted with the solvent front. Detection, 210 nm. Other conditions as in Fig. 1.

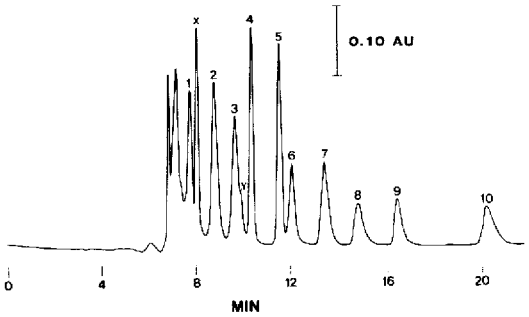

Fig. 4. Chromatogram of urine to which drugs have been added. Identity and concentration of drugs is given for Fig. 2. Sample, 500 μl of buffered urine; detection, 210 nm. For other conditions, see Fig. 1.

The detection limit of the system may be defined by the lowest concentration that yields a spectrum which can be matched against the stored spectral library. The library requires 10 mAU at λ_{max}, which will be different for different compounds. Using a 10-mAU cutoff at 210 nm, the minimal detectable amounts shown in Table IV were obtained. Obviously much smaller peaks can be observed. In practice, the detection limit for compounds eluted between caffeine and diazepam may be higher, owing to background contributions by caffeine and endogenous compounds. Benzoylecgonine (λ_{max} = 233 nm) is more readily detected at 235 nm, the secondary detection wavelength, where background contributions are reduced, as compared to 210 nm.

Suitability for toxicology screening

The College of American Pathologists distributes proficiency samples on a quarterly basis to toxicology laboratories for qualitative analysis. The 1988 program included a total of 77 compounds. To determine the utility of our method for toxicological screening, stock solutions of 71 drugs were prepared and added to urine to obtain concentrations of 2 mg/l. (Six volatile compounds were not evaluated.) Each sample was analyzed; the retention times and the absorbance at 210 nm and 235 nm

TABLE III

REPEATABILITY DATA FOR AUTOMATED MULTI-COLUMN SCREENING OF URINE

Compound	*Amount added (μg) to 500 μl urine*	*Retention time ± S.D. (min)*	*Peak height R.S.D. (%)*
Secobarbital	2.5	7.85 ± 0.003	2.7
Diazepam	1.0	8.89 ± 0.006	1.0
N-Ethylnordiazepam	2.0	9.73 ± 0.007	1.8
Amphetamine	2.5	10.43 ± 0.012	0.7
Methamphetamine	2.5	11.58 ± 0.009	0.6
Diphenhydramine	1.0	12.06 ± 0.012	0.7
Imipramine	1.0	13.29 ± 0.011	1.2
Morphine	1.0	14.61 ± 0.024	1.2
Chlorpheniramine	1.5	16.33 ± 0.023	1.2
Hydrocodone	1.0	19.90 ± 0.045	1.0

TABLE IV

LINEARITY DATA FOR AUTOMATED MULTI-COLUMN SCREENING OF URINE

Number of injections at each concentration = 3.

Compound	*Concentration range (mg/l)*	*Regression line*			*Detection limit*[a] *(mg/l)*
		Slope	*r*	*Intercept (mg/l)*	
Diazepam	0.3–25	$8.3 \cdot 10^{-5}$	0.9999	−0.04	0.3
Amphetamine	0.3–10	$4.5 \cdot 10^{-5}$	0.9999	−0.02	0.2
Methamphetamine	0.3–10	$4.8 \cdot 10^{-5}$	0.9999	−0.02	0.2
Diphenhydramine	0.3–25	$3.9 \cdot 10^{-5}$	0.9999	0.01	0.2
Imipramine	0.3–10	$4.4 \cdot 10^{-5}$	0.9999	0.03	0.3
Morphine	0.3–10	$9.4 \cdot 10^{-5}$	0.9991	−0.26	0.3
Hydrocodone	0.3–10	$1.2 \cdot 10^{-4}$	0.9997	−0.15	0.3

[a] Concentration producing a peak height of 10 mAU at 210 nm.

were recorded. The full spectra (195–375 nm) were also stored on hard disc. Of the 71 compounds injected 61 were retained on the sample preparation cartridges and produced symmetrical peaks which were separated by at least 0.5 min from the caffeine peak. (The proficiency program does not include caffeine.) Of the compounds that could not be analyzed, four were acids, four were neutral, and two were weak bases with no appreciable UV absorbance. Even weakly absorbing drugs such as phencyclidine produced observable peaks at concentrations of 50–100 μg/l, but their spectra could not always be matched against the stored spectra. The analytical system,

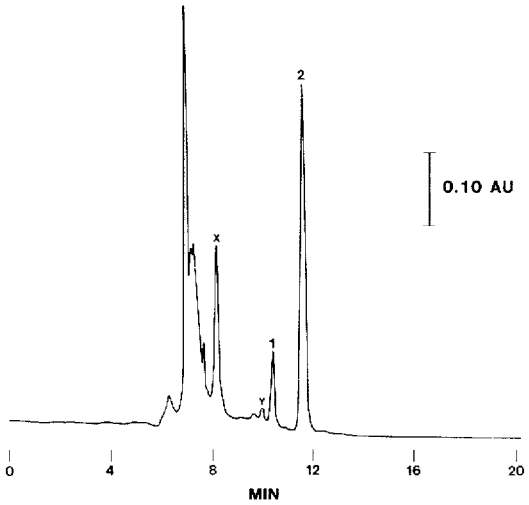

Fig. 5. Chromatogram of a urine specimen which was positive for amphetamines by thin-layer chromatography. Peak identities were confirmed by comparison with stored library spectra. Concentrations were determined by comparison with urines supplemented with known concentrations of drugs: 1 = Amphetamine (1.8 mg/l), 2 = methamphetamine (8.2 mg/l); detection, 210 nm. For other conditions, see Fig. 1.

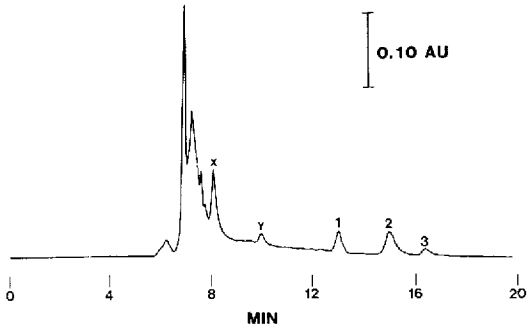

Fig. 6. Chromatogram of a urine sample which was positive for opiates by thin-layer chromatography. Peaks: 1 = monoacetyl morphine (0.6 mg/l), 2 = morphine (1.0 mg/l), 3 = codeine (0.2 mg/l). Detection, 210 nm. For other conditions, see Fig. 1.

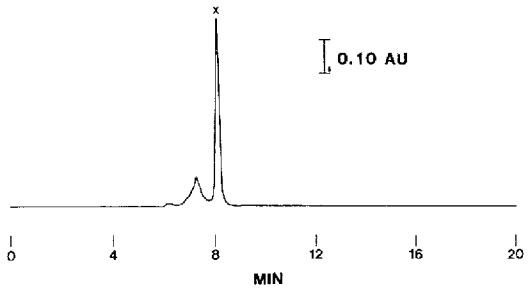

Fig. 7. Chromatogram of a drug-free urine sample. Detection, 235 nm. Note the absence of the endogenous peak Y. For other conditions, see Fig. 1.

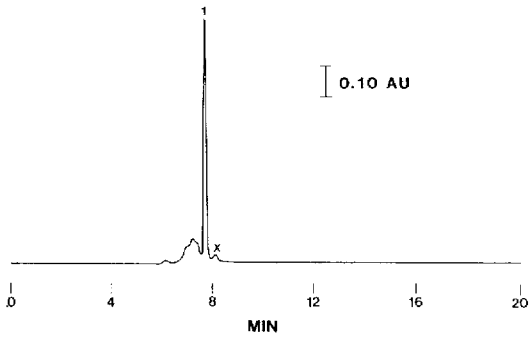

Fig. 8. Chromatogram of a urine specimen which was positive for benzoylecgonine by thin-layer chromatography. Peak: 1 = benzoylecgonine (22.4 mg/l). Note the small size of the endogenous peak X in this urine. Detection at 235 nm. For other conditions, see Fig. 1.

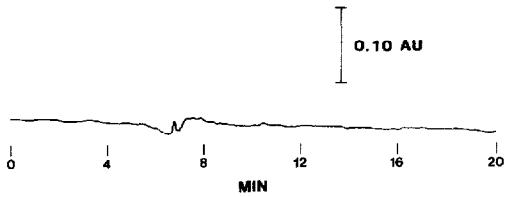

Fig. 9. Chromatogram of 500 μl of buffered drug-free serum. Detection, 210 nm. For other conditions see Fig. 1.

therefore, has broad applicability as a qualitative screening technique, and could be part of a confirmatory scheme for many drugs when concentrations exceed 300 μg/l.

Physiological samples may contain more than one drug, as well as their metabolites; these samples can be successfully analyzed only if the drug combination does not produce overlapping peaks. Because the elution order produces a separation by class, samples containing two different drug classes (*e.g.* amphetamines and opiates) are more readily analyzed than samples containing two drugs of the same class (*e.g.* imipramine and amitriptyline).

Analysis of biological specimens

Chromatograms were obtained for specimens previously analyzed by a thin-layer chromatographic technique (Figs. 5–8). Positive identification of benzoylecgonine, amphetamine, methamphetamine, 6-monoacetyl morphine, morphine, and codeine was possible by computerized evaluation of the chromatograms, using the library search routine.

Several techniques could extend the applicability of the system in toxicological analysis. Larger volumes of urine could be preconcentrated off-line by solid-phase or solvent extraction. Extraction of 5 or 10 ml urine might lead to a 10- to 20-fold reduction in detection limits.

Many drugs are difficult to analyze in urine because of extensive metabolism and conjugation. Serum samples would be more appropriate for such drugs, and blood is usually available for emergency toxicological screening. Fig. 9 shows the chromatogram obtained from a drug-free serum which was processed in the manner described above for urine. The nearly complete absence of background, including the endogenous peak observed (at 8 min) in almost all urines, suggests that serum samples may be especially useful for detection of benzodiazepines and barbiturates, which are eluted in the first few minutes of chromatography.

CONCLUSIONS

The use of two pre-columns for sample clean-up and two coupled analytical columns permitted the analysis of a broad range of drugs of toxicological interest. Elution under isocratic conditions eliminated the need for re-equilibration of the analytical columns and produced a constant spectral background, which simplified the storage and processing of UV spectra. Resolution was generally sufficient to permit separation of drugs from their hydroxylated and demethylated metabolites. Levels of 300 μg/l yielded peaks that were large enough for processing by a computerized library

search routine. Complete analysis required less than 30 min. This analytical system may be useful for toxicological screening in cases where very rapid identification (or exclusion) of a broad range of drugs is required.

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