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Separation of Standard Opiates and Their Analysis in Pharmaceutical and Illicit Preparations by Paired-Ion Reverse-Phase High-Pressure Liquid Chromatography

There are many methods of analysis at the disposal of a forensic scientist for the routine analysis of controlled drug substances. However, the main objective has always been to introduce new, versatile techniques with high efficiency, selectivity, and excellent precision.

Gas chromatography (GC) for qualitative and quantitative analysis and thin-layer chromatography (TLC) for qualitative analysis are the currently available rapid methods. High efficiency, speed, reproducibility, and the ease of quantitation make GC one of the most attractive techniques for screening and detection of common drugs of abuse. However, there are many limitations to this method; substances to be analyzed should be volatile, thermally stable, and, furthermore, should not be highly polar. These limitations are often overcome by the chemical modification of the sample components. Without such modification, it is estimated that only 20% of the known organic compounds can be analyzed by GC [*1*]. Thin-layer chromatography is a relatively inexpensive and fast method but it is difficult to automate, lacks reproducibility, and is unsuitable for quantitation. The visualization of the substances by chemical reagents on TLC plates usually makes the recovery impossible.

High-pressure liquid chromatography (HPLC) is the answer to the problems associated with GC and TLC. It is normally conducted at room temperature, making the method suitable for an increasing number and types of compounds. There is no special procedure necessary to handle nonvolatile, thermally labile, and highly polar compounds for analysis with LC. The pure samples can be easily collected for further analysis by infrared or mass spectrometry. It is the result of recent developments occurring in many different areas [*1-5*]. The gains in versatility are attributed to developments in columns, high-surface-area packings, high-pressure constant delivery pumps, and more sensitive detectors. There are basically four modes of mechanisms in liquid chromatography: liquid-solid absorption, liquid-liquid partition, ion exchange, and gel permeation. The usefulness of HPLC employing these separation mechanisms in the area of forensic science has been extensively investigated [*6-18*]. The separations by these chromatographic procedures seem to be characterized by their inability to separate a large variety of structurally related compounds on a single column.

The reverse-phase paired-ion technique is the most recent development in HPLC [*19-21*]. This technique involves the use of the nonpolar stationary phase and the liquid mobile

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phase with an appropriate counter ion added to it. The reversible ion-pair complexes are formed between the sample components and the counter-ions. Then the separation of the sample components occurs depending on the differences in the rates of adsorption and desorption of ion-pair complexes on the surface of the stationary phase. The paired-ion chromatography (PIC) overcomes the problems of precise pH control and reproducibility encountered in ion-exchange chromatography. By the judicious choice of counter-ions, PIC is capable of simultaneous analysis of acids, bases, and neutral compounds [22].

The application of this technique to the analysis of only the standard solutions of major opium alkaloids and some of their derivatives on a reversed phase column has been recently reported by Olieman et al [23] and Lurie [22]. The present investigation was undertaken for the separation and the detection of twelve opiates of forensic science interest and for the quantitative determination of heroin in clandestine preparations. This study was also extended to include the analysis of opiates in pharmaceutical drugs without any prior solvent extraction and purification. Two parameters, retention times and the ratios of the absorbance peak heights recorded at 254 and 280 nm, were used to identify the opiates in a mixture. A preliminary report of this work has been presented at the 30th Annual Meeting of the American Academy of Forensic Sciences [24].

Materials and Methods

A Waters Associates liquid chromatograph equipped with the components listed in Table 1 was used in conjunction with a 10-mV recorder (Houston Instrument, Houston, Tex.). The variable wavelength detector was operated at 280 nm because of the high sensitivity of detection of opiates around this wavelength [25]. The two detectors, Models 450 and 440, were connected in series; the dead volume between the two detectors was negligible because of the extremely small diameter of stainless steel tubing (0.229 mm [0.009 in.] inside diameter). Column effluents were therefore monitored almost simultaneously at 280 and 254 nm. The fluid volume of each flow-cell in detector Model 450 was 8 μl.

The stationary phase was a prepacked micro-Bondapak® C_{18} column (Waters Associates); this column along with the appropriate mobile phase could provide the high efficiency necessary for the separation of complex mixtures of opiates, the structures and basicity of which were closely related. The micro-Bondapak C_{18} has a monolayer of octadecylsilane chemically bonded to micro-Porasil® beads of an average particle size of 10 μm.

The mobile phase consisted of methanol ("Spectranalysed," Fisher Scientific Co.) either with 0.01*M* PIC A solution of pH 7.5 or with 0.01*M* PIC B-7 solution of pH 3. PIC A is an aqueous solution of tetrabutyl ammonium phosphate and PIC B-7 is a heptane sulfonic acid solution containing acetic acid. The concentrated solutions of these reagents were obtained from Waters Associates and used according to manufacturer's instructions. Alternatively, they can be prepared from tetrabutyl ammonium hydroxide and heptane

TABLE 1—*Summary of liquid chromatographic parameters.*

Instrument	Waters Associates, Milford, Mass., equipped with Model 660 solvent programmer, Model 6000 A delivery system, Model U6K injectors, Model 440 fixed wavelength of 254 nm, and Model 450 variable wavelength
Column	micro-Bondapak C_{18} (30 cm by 4 mm inside diameter)
Mobile phase	methanol with PIC A solution (pH 7.5) and PIC B-7 solution (pH 3.0)
Temperature	ambient
Flow rate	2 ml/min
Injector volume	2 to 5 μl
Concentration of sample	2 to 5 mg/ml of water or methanol
Chart speed	25 mm (1 in.)/5 min

sulfonic acid (Eastman Kodak Co.), respectively. The tetrabutyl ammonium hydroxide solution was adjusted to pH 7.5 with 85% phosphoric acid; the PIC B-7 solution of heptane sulfonic acid contained 1% (v/v) acetic acid and recorded a pH of about 3. The pH values were adjusted so that the sample components were present in their ionic forms. The solvents used were filtered through Millipore filters with a pore size of 0.45 μm to remove suspended particles and degassed under vacuum just prior to use. The pH values of the individual solutions were measured with a Beckman pH meter (Model Expandomatic SS-2). After being adjusted to the required pH, PIC reagent solution was mixed automatically with methanol in a reference manifold assembly of the M 6000 A pump as per conditions set on solvent programmer. The exact composition of the mobile phase is given in the legend of each chromatogram.

The opiates and other standards (aspirin, phenacetin, caffein, and hexabarbital) used conformed to U.S. Pharmacopeia specificastions. Tylenol® No. 3 (McNeil Labs), Tabloid Brand No. 2 (Burroughs Wellcome), Emprazil-C® (Burroughs Wellcome), Phenergan® expectorant C (Wyeth Labs), Percodan® (Endo Labs), and Percobarb® (Endo Labs) were the drug products used in this study. Water was used to advantage as a solvent to dissolve the opiate salts present in these pharmaceutical preparations. Aqueous solutions of the pure opiates' salts were used as standards.

A Precision Sampling Corp. Series B-110, 0- to 25-μl syringe was used for injection purposes. The 25-gage syringe needle was 50 mm long by 0.51 mm outside diameter.

The column was washed with water followed by methanol for about half an hour at the end of the day for proper maintenance of the column.

Results and Discussion

Separation and Identification of Opiates

The identification of the standard opiates was based on two parameters: retention times and the ratios of absorbance peaks recorded at 254 and 280 nm, where 254 nm is a fixed wavelength on one ultraviolet (UV) absorbance detector. The selection of 280 nm on the other UV-visible detector was due to the high characteristic absorbance of opiates around this wavelength [25]. Since the molar absorptivity at the wavelength of detection is a measure of the sensitivity of detection, the detectable amount for a particular compound could be on the order of nanograms depending on the selection of wavelength and the setting of highest sensitivity on the instrument.

The separation techniques normally depend on the retention time of an individual compound, usually coupled with other chemical reactions as a means of tentative identification. In the present study, the additional parameter, the absorbance ratio, provided a powerful means of supplementing the retention time as a criterion of identification; this parameter could be measured with great accuracy and reproducibility at different absorbance sensitivities, as shown in Table 2. For each solvent system studied, each compound was injected to determine the individual retention time and the absorbance ratio. Then the mixture of many opiates was injected to determine the degree of resolution of each component. The same procedure was applied for illicit preparations and pharmaceutical drugs.

Basically, two systems differing in pH and in the composition of the mobile phase were used; the results of studies of twelve opiates under these conditions are given in Table 3. These results show that there is no separation between morphine, oxymorphone, and noroxymorphone with Solvent b (35% methanol with PIC B-7 solution) (after 10 min, the concentration of methanol was increased to 55%) and very poor separation between monoacetylmorphine and oxycodone. On the other hand, morphine, oxymorphone, and noroxymorphone separate well with Solvent a (47% methanol with PIC A), as do mono-

TABLE 2—*Absorbance peak ratios[a] between the values recorded at 254 and 280 nm at different sensitivities in 35% methanol with PIC B-7 solution.*

Compound	Detector (254 nm), 0.2 Absorbance/Detector (280 nm), 0.2 Absorbance; for Full Scale	Detector (254 nm), 0.05 Absorbance/Detector (280 nm), 0.04 Absorbance; for Full Scale
Morphine	0.75 ± 0.005	0.76 ± 0.010
Monoacetylmorphine	0.59 ± 0.010	0.60 ± 0.010
Heroin	0.24 ± 0.005	0.24 ± 0.010
Oxymorphone	0.85 ± 0.010	0.86 ± 0.012
Noroxymorphone	0.87 ± 0.007	0.85 ± 0.010
Oxycodone	0.85 ± 0.004	0.85 ± 0.012
Hydrocodone	0.76 ± 0.010	0.77 ± 0.010
Nalorphine	0.79 ± 0.005	0.77 ± 0.010
Codeine	1.10 ± 0.010	1.12 ± 0.015
Acetylcodeine	5.25 ± 0.100	...
Dilaudid®	0.77 ± 0.008	0.77 ± 0.010
Papaverine	~10	...

[a] At least five injections were made for each computation.

acetylmorphine and oxycodone. However, the distinction between morphine and oxymorphone and noroxymorphone can be made based on absorbance peak ratios with both these solvents.

The elution pattern of eight resolved peaks with Solvent b is given in Fig. 1. Oxycodone and hydrocodone were injected separately from the eight opiates for the sake of clarity and their separation is shown in Fig. 2. Heroin and papaverine (thebaine and narcotine [noscapine] are not shown) are eluted from the column only when a solvent gradient increasing to a higher concentration of methanol is applied (Fig. 1). Lurie [*22*] reported similar results using the same mobile and stationary phases as were used in this study.

Twitchett and Moffat [*26*] evaluated the micro-Bondapak C_{18} column for the analysis of a wide variety of drugs with aqueous methanolic solution at different pH. Excellent efficiency and resolution for acidic and neutral drugs was given by this column but poor

TABLE 3—*Absorbance peak ratios between the values recorded at 254 and 280 nm (Ratio 254/280) and the retention times in minutes (R_{min}) of opiates.[a]*

Compound	R_{min}		Ratio 254/280	
	Solvent b	Solvent a	Solvent b	Solvent a
Acetylcodeine	2.5	...	5.25	...
Morphine	5.0	7.0	0.75	0.72
Oxymorphone	5.0	5.0	0.85	0.94
Noroxymorphone	5.2	3.8	0.87	0.84
Dilaudid	5.8	...	0.77	...
Nalorphine	7.1	...	0.79	...
Codeine	8.3	12.5	1.10	1.05
Monoacetylmorphine	10.1	9.0	0.59	0.54
Oxycodone	10.4	10.5	0.85	0.84
Hydrocodone	11.8	R	0.76	...
Heroin	15.4	...	0.24	...
Papaverine	16.7	...	10.1	9.9

[a] Solvent a = 47% methanol with PIC A, flow rate 2 ml/min; Solvent b = 35% methanol with PIC B-7 for 10 min, then methanol concentration was increased to 55%, flow rate 2 ml/min; R = retained on the column.

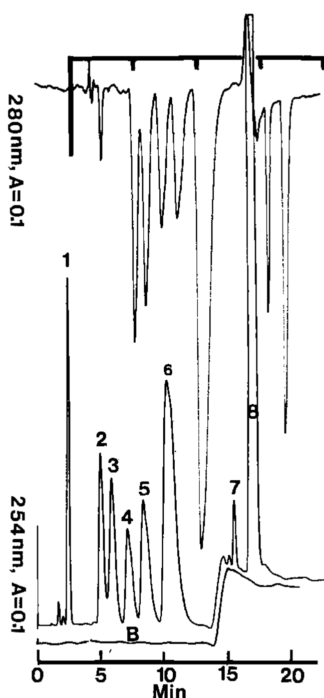

FIG. 1—*Separation of aqueous opiate solutions: acetylcodeine* (1), *morphine* (2), *Dilaudid* (3), *nalorphine* (4), *codeine* (5), *monoacetylmorphine* (6), *heroin* (7), *and papaverine* (8). *Solvent gradient: 35% to 55% methanol with PIC B-7 solution (35% methanol was used for 10 min and then its concentration was increased to 55%).* (A) = *absorbance for full scale and* (B) = *baseline.*

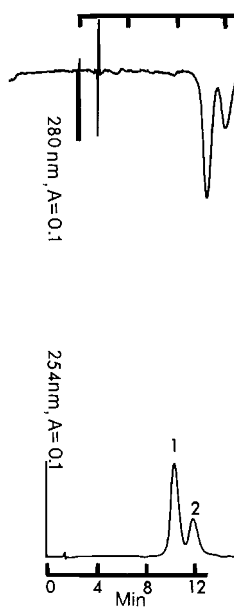

FIG. 2—*Chromatogram of an aqueous sample solution of oxycodone* (1) *and hydrocodone* (2) *with 35% methanol and PIC B-7 solution.*

efficiency was seen by these authors with most basic drugs. However, in our study, the use of PIC reagents with the same reversed phase column allowed the separation of basic drugs, the structures of which were closely related (Figs. 1 and 2).

Analysis of Heroin in Clandestine Preparations

Heroin is usually found in clandestine preparations as a white or brown powder adulterated with diluents. Monoacetylmorphine is present in most of these powders either as a by-product of heroin synthesis or as a result of hydrolysis. Knox and Jurand [*12*] reported that heroin undergoes rapid hydrolysis in a buffered solution at room temperature and the hydrolysis is complete in about 4 h. In clandestine use heroin powder is often heated in a bottle cap to liquify it for injection. In such a case, monoacetylmorphine will be present in great quantity along with a small proportion of morphine [*12*]. It was therefore important to determine the optimal isocratic conditions in order to analyze and to quantitate heroin in illegal seizures without interference from incipients (morphine and monoacetylmorphine) and excipients (such as sugars, quinine, procaine, and methapyrilene). Figure 3 illustrates the separation of nalorphine, monoacetylmorphine, and heroin. Because these compounds have baseline separation, nalorphine could be used as an internal standard for quantitative determination of heroin without any interference from monoacetylmorphine and morphine (morphine elutes closer to void volume). The areas under the heroin peaks were measured with a Hewlett-Packard integrator, Model 3370 A. However, the quantitation based on peak heights was equally accurate. The detector response was linear for heroin in the concentration range (2 to 10 μg) used in this study.

Quinine, methapyrilene, and procaine did not elute at the place of compounds of interest. In a recent report [*22*] these diluents were shown to separate from major opium alkaloids. Sugars did not interfere by virtue of their nonabsorbing nature. Examples substantiating these facts are given in Fig. 4, which represents the chromatograms of heroin residue

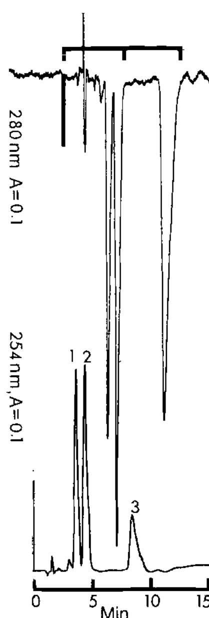

FIG. 3—*Chromatogram of an aqueous sample solution of nalorphine* (1), *monoacetylmorphine* (2), *and heroin* (3), *with 46% methanol and PIC B-7 solution.*

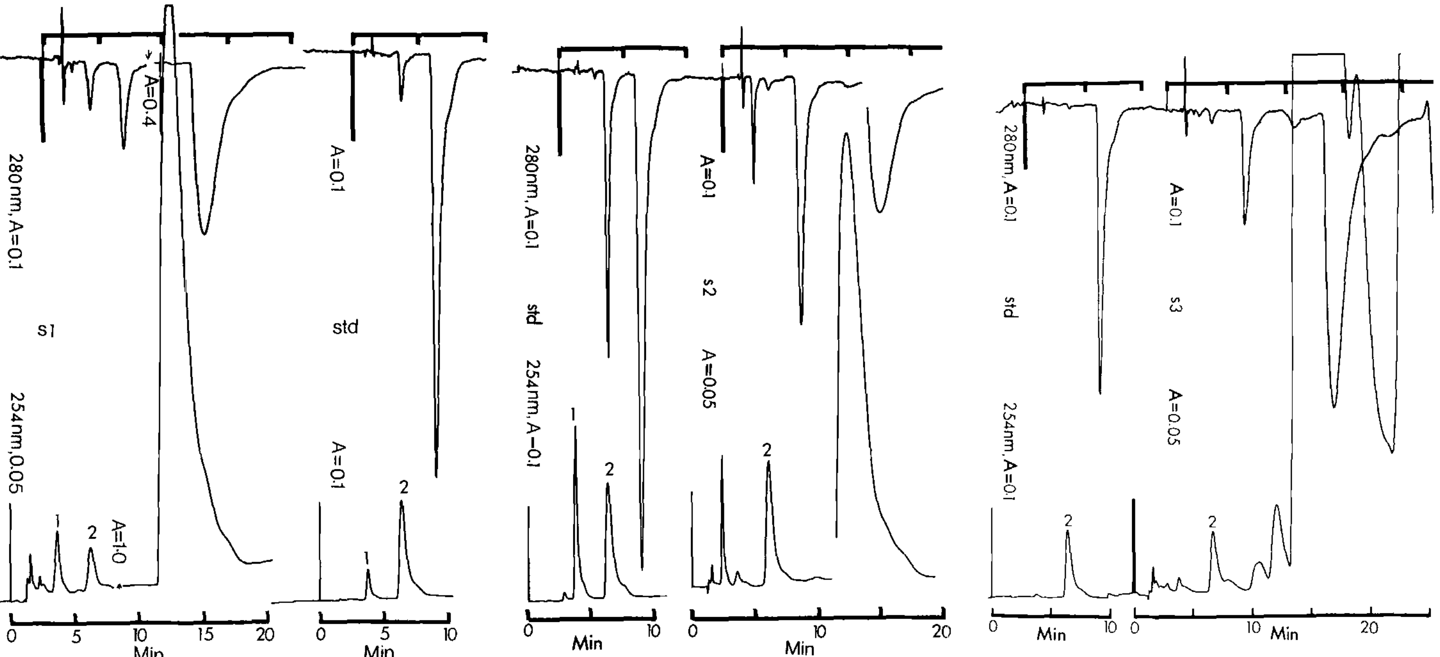

FIG. 4—*Chromatograms of methanol solutions: a burnt bottle cap contents (s1), illicit white powder (s2), and illicit brown powder (s3). Heroin (2) and monoacetylmorphine (1) are identified in the s1, s2, and s3 chromatograms by comparison with standard (std) solutions. Mobile phase: 46% methanol with PIC B-7 solution.*

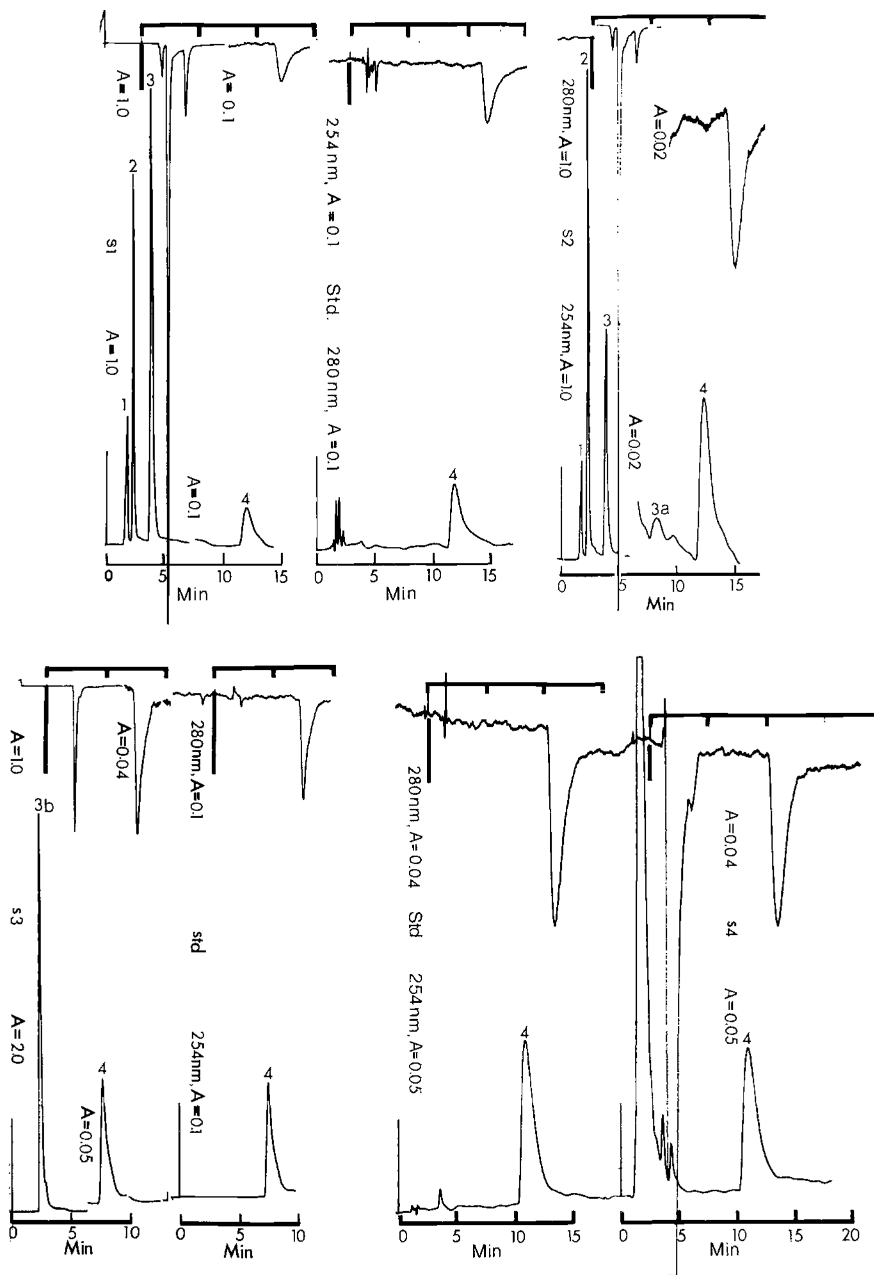

FIG. 5—*Chromatograms of solutions*: *Tabloid Brand No. 2* (s1), *Emprazil-C* (s2), *Tylenol No. 3* (s3), *and Phenergan expectorant C* (s4). (*For each analysis, one quarter of a tablet was dissolved in 1 ml of water*; *Phenergan expectorant C was diluted 1:1 with methanol*). *Codeine* (4) *was identified in the s1, s2, s3, and s4 chromatograms by comparison with standard* (std) *solutions. Aspirin* (1), *caffeine* (2), *phenacetin* (3), *pseudoephedrine* (3a), *and acetaminophen* (3b) *were identified separately* (*comparison is not shown*). *Mobile phase: 47% methanol with PIC A solution for s1, s2, and s4*; *35% methanol with PIC B-7 solution for s3.*

in a burnt bottle cap (s1) and heroin and monoacetylmorphine (s2 and s3) in illicit white and brown powders. The presence of these narcotics was established by measuring the retention times and the absorbance ratios, which were compared with the values of standard solutions of these drugs.

Analysis of Opiates in Prescription Drugs

This study was also extended to include the analysis of the several multicomponent prescription drug products that are complex mixtures of various active ingredients and excipients. The common active components in Tabloid Brand No. 2 and Emprazil-C are aspirin (A), caffeine (C), phenacetin (P), and codeine. In addition, Emprazil-C contains pseudoephedrine. Similarly, Percodan (not shown in Fig. 6) and Percobarb have oxycodone in addition to the analgesic ingredients APC. Hexobarbital is present only in Percobarb capsules. Tylenol No. 3 has two active ingredients: codeine and acetaminophen. The components of Phenergan expectorant C are codeine, promethazine hydrochloride, potassium guaiacolsulfonate, sodium citrate, and a trace of chloroform. Codeine and oxycodone were successfully separated and identified from these pharmaceutical preparations and their elution patterns are given in Fig. 5 (s1, s2, s3, s4) and Fig. 6 (s1). Hexobarbital eluted at the position of phenacetin (Peak 3, Fig. 6). This result, however, did not interfere with the analysis of oxycodone, which eluted at a later time.

In the past, GC has been attempted for separation and analysis of many analgesics but has not been very successful without the prior formation of a derivative, necessitated by either thermal degradation or the nonvolatile nature of these compounds [*27-30*]. Since these analyses with HPLC were performed at ambient temperature, no problems resulting from thermal degradation, as had been encountered in GC, were possible.

Paired-ion chromatography with a reversed-phase column proved to be a versatile

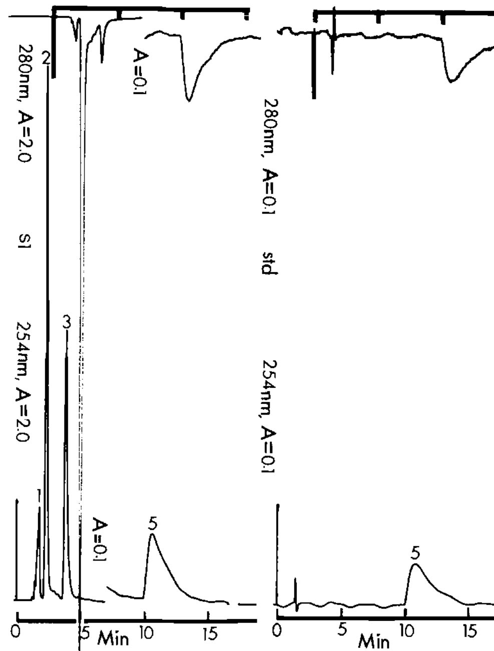

FIG. 6—*Chromatogram of a Percobarb solution* (s1). (*One half of a capsule was dissolved in 1 ml of water*). *Oxycodone* (5) *was identified in the s1 chromatogram by comparison with standard* (std) *solution. Aspirin* (1), *caffeine* (2), *and phenacetin or hexobarbital* (3) *were identified separately* (*comparison is not shown*). *Mobile phase: 47% methanol with PIC A solution.*

system that provided the high efficiency, specificity, and sensitivity required for the analysis of these drugs of abuse with minimum sample handling. In this study, the use of the additional parameter, the UV absorbance ratio, supplementing the retention time as a means of identification, had minimized the probability of mistakes occurring because of another compound eluting with the same retention time.

Summary

The separation of standard opiates in a mixture and their analysis in clandestine and pharmaceutical preparations were accomplished by PIC on a micro-Bondapak C_{18} column. The identification of the opiates was based on two parameters: retention times and the ratios of absorbance peaks recorded at 254 and 280 nm. No prior clean-up procedure of samples was required for analysis by this method. Baseline separation of drug components in clandestine and in pharmaceutical preparations made this method suitable for their quantitation.

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