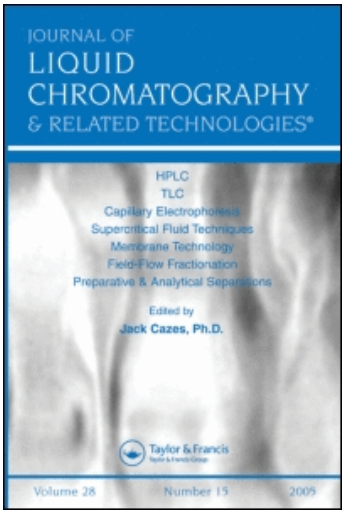


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# Determination of Common Analgesics in Serum and Urine by Liquid Chromatography/Electrochemistry

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# DETERMINATION OF COMMON ANALGESICS IN SERUM AND URINE BY LIQUID CHROMATOGRAPHY/ELECTROCHEMISTRY

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## ABSTRACT

A reverse phase LC method with amperometric electrochemical detection is described for determination of several common analgesics in blood serum and urine. The retention properties as a function of organic mobile phase modifier and pH are graphically illustrated and discussed. Electrochemical properties of the analgesics and possible internal standards are also presented. Step-gradient elution was used to facilitate simultaneous determination of analgesics with a wide range of hydrophobicities. Parallel dual-electrode detection with two glassy carbon working electrodes enables acetaminophen to be selectively determined in the same chromatographic run at +0.75 V while several other analgesics are determined at +1.15 V.

## INTRODUCTION

Analgesics are a group of readily available and widely consumed pharmaceutical products. Acetaminophen (APAP), salicylate (SAc), salicylamide (SAm), phenacetin (Phen), methyl salicylate (MeS), naproxen (NPX), and codeine (Cod) (see Figure 1) are among the most common. They may be self-administered and/or clinically prescribed



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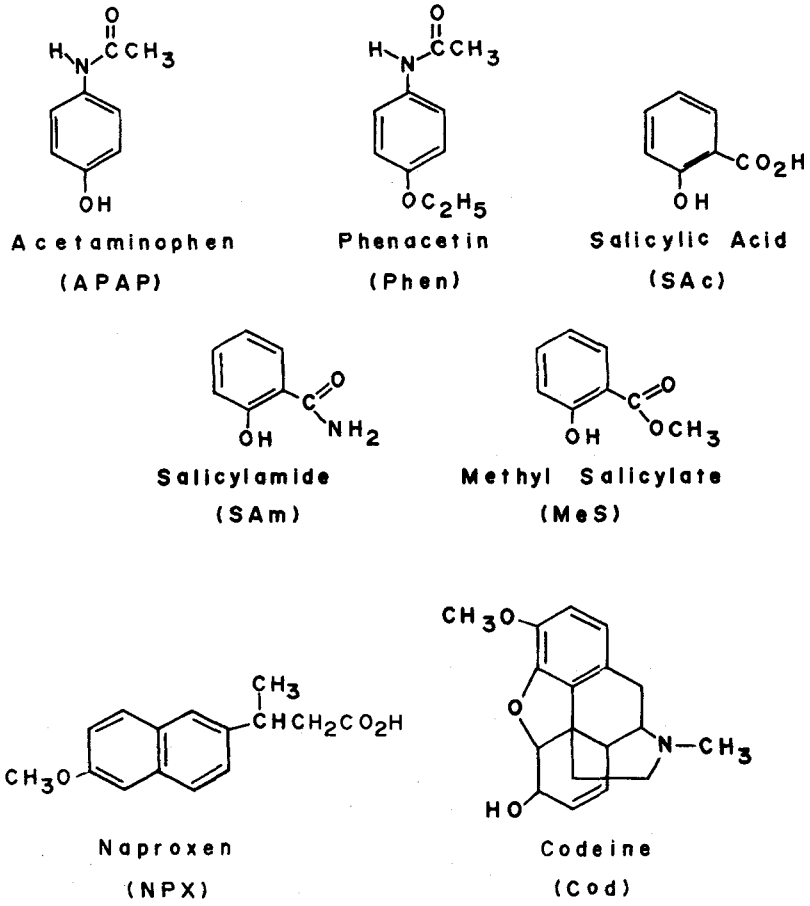


FIGURE 1. Structural formulas of the common analgesics investigated.

in a variety of combinations and dosage levels (1,2). Their determination in biological fluids is important for toxicological, clinical, drug interaction, and pharmacokinetic studies.

Liquid chromatographic procedures have been described for determining many of these compounds in blood serum or plasma (3-10) and in dosage formulations (11). Simultaneous deter-

mination of several common analgesics has seldom been presented and determinations in urine have been largely ignored. The majority of LC separations reported employ UV detection and are thus subject to interference by many other common drugs. Fluorescence detection has also been reported (12,13) but lacks general applicability. Unlike some other drugs, the common analgesics shown in Figure 1 are electrochemically active at analytically useful potentials and are therefore amenable to the selectivity and sensitivity of electrochemical (EC) detection. Determination of acetaminophen in serum by LCEC has previously been demonstrated (14).

Investigations were undertaken to evaluate the electrochemical and chromatographic properties of these analgesic compounds and of several possible internal standards. Step-gradient elution was evaluated to determine its usefulness and compatibility with EC detection. Dual electrode detection is shown to provide increased versatility by allowing simultaneous determination of early eluting APAP at a significatly lower and thus more selective detector potential.

Simple, rapid sample preparation was employed to provide methods which would facilitate routine use of LCEC in these determinations.



## EXPERIMENTAL

### Reagents

Acetaminophen, salicylic acid, and salicylamide were obtained from Sigma Chemical Co. (St. Louis, MO); codeine and methyl salicylate from Mallinckrodt (Paris, KY); naproxen was a generous gift from Syntex (Palo Alto, CA); phenacetin (4'-ethoxy-acetanilide), 2-acetamidophenol, and 3-methylsalicylic acid from Aldrich (Milwaukee, WI); and p-hydroxyphenylpropionic acid and p-methoxy-acetanilide from Pfaltz and Bauer (Stamford, CT). N-propionyl-p-aminophenol (PrPAP) was synthesized by conventional methods

(15). Water was double distilled and methanol was single distilled technical grade. All other chemicals were reagent grade.

## Apparatus

Cyclic voltammetry was performed with a CV-1A cyclic voltammetry instrument from Bioanalytical Systems, Inc. (BAS) (West Lafayette, IN).

The liquid chromatograph consisted of a Milton Roy single-piston pump, a Rheodyne model 70-10 injector with a 20 µL loop, and a Biophase 5 µm $C_{18}$ column (25 cm x 4.6 mm) (BAS). Electrochemical detection was performed with a dual-electrode system in the "parallel-adjacent" mode by use of two modified BAS LC-4A controllers and a BAS dual-electrode thin-layer cell with glassy carbon working electrodes and a Ag/AgCl reference electrode. A Rheodyne low pressure 4-way rotary valve was placed upstream of the pump to allow alternate selection of two mobile phases. Column temperature was controlled with a BAS LC-23 column heater and LC-22 temperature controller.



## Cyclic Voltammetry

Cyclic voltammetric data for APAP, SAc, SAm, Phen and MeS were obtained at concentrations of 1 mM in 25% methanol with pH 4.0 acetate (0.1 M) and 0.1 M $KNO_3$ as buffered electrolyte. Naproxen was scanned at 2 mM in 40% methanol: 60% (v:v) pH 5.0 acetate (0.1 M). Codeine was evaluated at 5 mM in 50% methanol: 50% pH 4.5 acetate buffer (0.05 M) containing 0.1 M $NaClO_4$. The scan rate was 150-200 mV/sec.

## Standard and Recovery Solutions

Stock analgesic solutions were prepared containing 1 mg/mL of each compound in 70% methanol with ethyl paraben (EtP) and PrPAP included as internal standards. Aliquots of pooled serum and urine were spiked with the appropriate volume of stock

solution to achieve the desired concentration levels. Working standards were prepared by addition of stock solution to an equivalent aliquot of water and processed identically.

## Sample Preparation

Serum samples were prepared by mixing 500 µL of spiked serum with 500 µL of acetonitrile in 1.5 mL Eppendorf capped centrifuge tubes, followed by centrifugation in an Eppendorf centrifuge (Brinkmann Instruments, Westbury, N.Y.) for five minutes. To 500 µL of the supernatant was added 500 µL of 0.1 M (pH 3.2) monochloroacetate (MCA) buffer and 100 µL of 1.0 M phosphoric acid. For samples in which codeine was to be determined, the supernatant was diluted with 0.1 M (pH 7.0) phosphate buffer. The samples were then filtered through 0.2 µm Nylon-66 filter membranes with a Swinnex syringe filter assembly (Millipore Corp., Bedford, MA).

Urine samples were prepared in a similar manner, but were not filtered. At higher concentrations, some additional dilution with mobile phase was employed to remain in a more desirable detector sensitivity range. For acetaminophen determinations, 500 µL samples were mixed with 500 µL of 1 M (pH 7.0) phosphate in a small test tube and extracted with 1.0 mL of ethyl acetate by vortex mixing. A 500 µL portion of the organic extract was evaporated under nitrogen and the residue reconstituted in 500 µL mobile phase.

## Liquid Chromatography

The retention properties of several compounds were evaluated versus organic modifier strength by varying the percentage methanol (v/v) in a mobile phase containing 0.1 M acetate buffer at pH 5.0. Retention versus pH was determined for NPX, SAc, and Cod by using MCA, acetate, and phosphate buffers to vary the pH of a 50% methanol mobile phase.

Serum samples were initially evaluated under isocratic conditions with a mobile phase containing 0.05 M ammonium MCA (pH 3.2) and 0.1 M $NaClO_4$ in 50% methanol:50% water (v:v). For step changes in eluent strength, eluents A and B contained 40 and 60 percent methanol, respectively, with the same molar concentrations of buffer and electrolyte. The stepwise gradient change was effected by manual switching from eluent A to B at a consistent time (5 min.). Serum recovery samples were run via gradient elution with dual-electrode detection at +0.75 and +1.15 V and at room temperature to simultaneously determine all analgesics except codeine. Codeine was determined isocratically with 50% methanol:50% pH 7.0, 0.2 M phosphate buffer, a column temperature of 30°C, and an applied potential of +1.12 V. A flow rate of 1.5 mL/min. was used for all studies.

Urine recovery samples were run with gradient elution for determination of SAm, SAc, Phen, MeS, and NPX. APAP was determined with eluent A and an applied detector potential of +750 mV following the quick extractive cleanup described above. Codeine in urine was determined with a mobile phase containing pH 5.8 phosphate (0.1 M) in methanol:water (45:55, v/v) and a column temperature of 45°C.

Analyte and internal standard responses were quantitated by measuring peak height. Relative recovery values were calculated by comparing analyte/internal standard response ratios of spiked samples against aqueous standards of similar concentrations. Samples were usually bracketed by standards for every 3 to 5 injections.

## RESULTS AND DISCUSSION

### Electrochemical Properties

Half-wave potentials from chromatographically assisted hydrodynamic voltammograms (HDV's) of the analgesic compounds (see Figure 1) and several possible internal standards are

TABLE 1

Hydrodynamic Voltammogram (HDV) Half-wave Potentials ($E_{1/2}$) for Common Analgesics and Possible Internal Standards*

| ANALGESICS | $E_{1/2}$ (VOLTS) |
|---|---|
| Acetaminophen | 0.62 |
| Phenacetin | 1.08 |
| Salicylamide | 1.03 |
| Salicylic Acid | 1.09 |
| Methyl Salicylate | 1.10 |
| Naproxen | 0.86 |
| Codeine | 1.12 |
| INTERNAL STANDARDS | |
| N-Propionyl-p-aminophenol | 0.62 |
| 2-Acetamidophenol | 0.65 |
| p-Methoxyacetanilide | 1.08 |
| Ethyl Paraben | 1.09 |
| p-Hydroxyphenylpropionic Acid | 0.92 |
| 3-Methylsalicylic Acid | 1.09 |

* LC mobile phase conditions: pH 4.5 acetate (0.1 M) with variable percent methanol, except for codeine which was evaluated in pH 7.0 phosphate (0.1 M) with 50% methanol.



listed in Table 1. Normalized HDV's of the analgesics are illustrated in Figure 2 and representative cyclic voltammograms (CV's) are shown in Figure 3.

Acetaminophen and phenacetin are substituted aminophenols whose electrochemical properties have been previously investigated (15). APAP is oxidized at modest potentials, whereas Phen is shifted to somewhat higher potentials due to ethyl substitution of the phenol. Although SAc, SAm, MeS, and EtP (I.S.) represent ring-substituted phenols and require even greater anodic potentials, they are well within the analytically useful range. Naproxen, a substituted naphthol, is oxidized at a lower potential

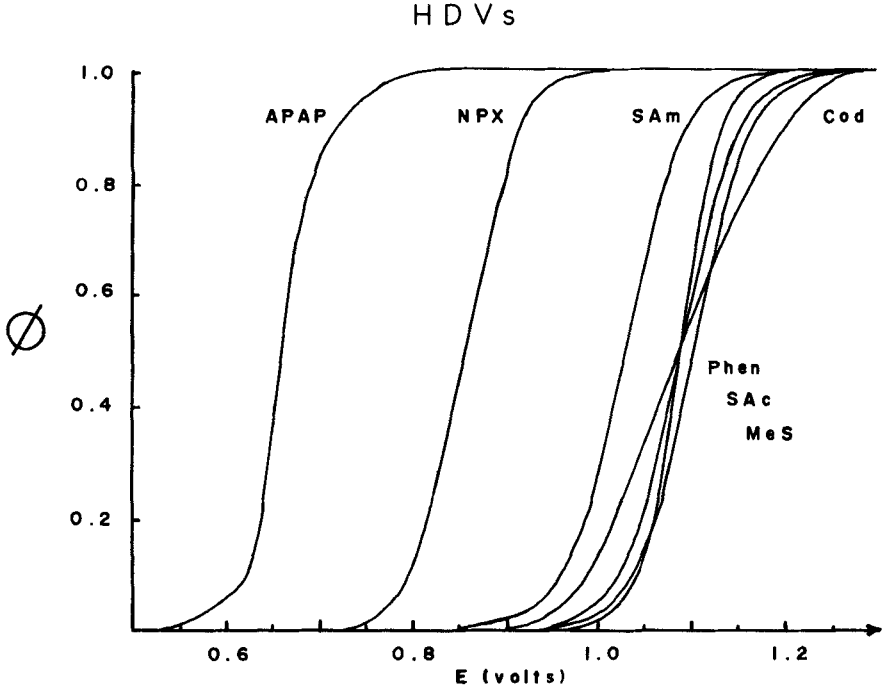


FIGURE 2. Normalized hydrodynamic voltammograms (HDV's) of analgesic compounds. LC mobile phase conditions same as Table 1.



than free phenols. The extensive ring conjugation is apparently responsible for this unexpected behavior.

Codeine is more difficult to oxidize but still may be determined at an advantageous potential. There is some evidence to support oxidation at the tertiary nitrogen (17,18) but the presence of a disubstituted catechol group suggests possible participation of this site. The appearance of two anodic waves in its CV indicates that both sites may be involved at adjacent potentials.

Chromatographic Properties

As shown in Figure 4, the desired analytes tend to elute with a wide range of retention times. APAP and its correspond-

ing internal standard (PrPAP) which elute early, can be detected with greater selectivity due to their significantly lower oxidation potentials.

The retention of SAc, NPX, and Cod is strongly pH dependent as shown in Figure 5. In order to sufficiently increase the retention of SAc relative to SAm and endogenous components, it was necessary to use a mobile phase of pH 3.2. At this pH, SAc (ca. $pK_a$ = 3.0) is partly undissociated and thus more strongly retained. NPX, however, is predominately unionized and exhibits strong lipophilic behavior. Codeine is almost completely cationic at this pH and elutes with the endogenous milieu of biological samples. At values near pH 6 and above, the retention of Cod is sufficiently increased to provide separation from most endogenous compounds. Endogenous electroactive urine components tend to be predominately anionic near neutral pH, and a general reversal of retention properties therefore occurs. The anionic nature of such urine components was demonstrated by observing their strong retention on an anion-exchange minicolumn.

Simultaneous determination of all the analgesics does not appear readily feasible due mainly to the disparate properties of codeine. However, the remainder can be determined in a single chromatographic run. Employment of gradient elution to alleviate the problem of diverse retention times is illustrated and discussed below.

## Internal Standards

The use of appropriate internal standards facilitates accurate quantitation while eliminating stringent volumetric transfers during sample preparation. Compensation is also made for slight changes in injection volume and chromatographic parameters. Several compounds were evaluated for suitable electrochemical and chromatographic properties. EtP and PrPAP were chosen as most appropriate for these investigations. The additional com-

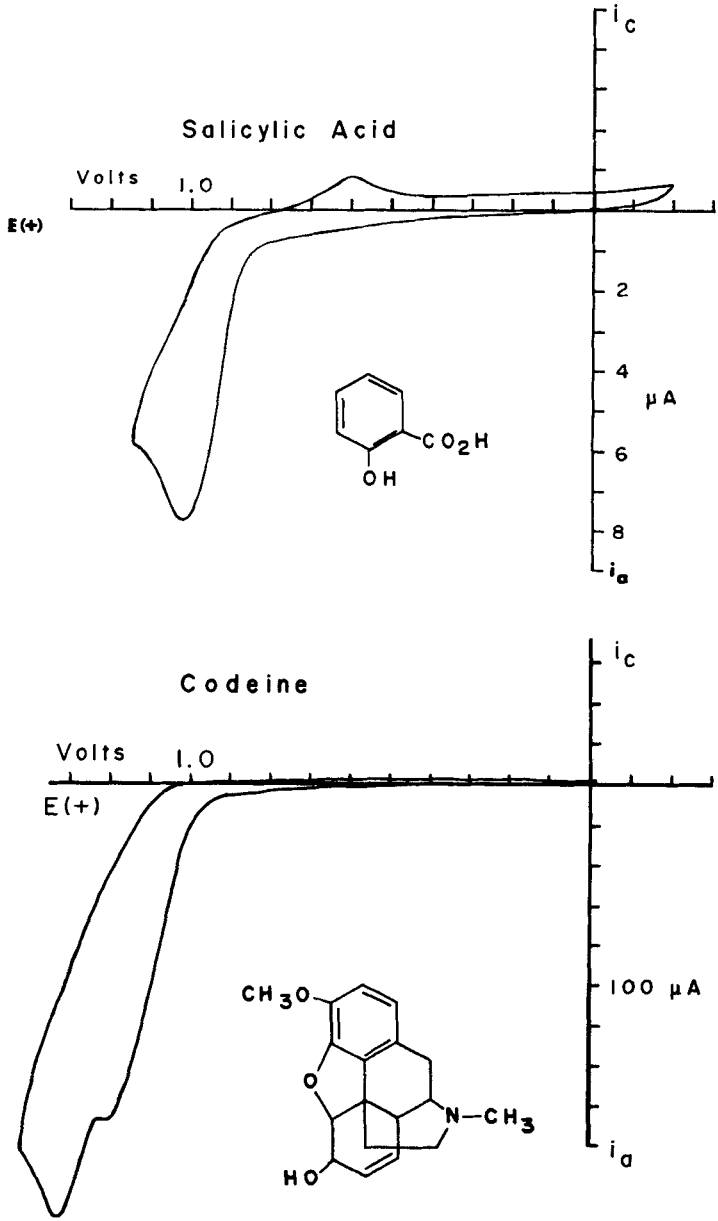


FIGURE 3. Cyclic voltammograms of acetaminophen, salicylic acid, naproxen, and codeine. See text for conditions.

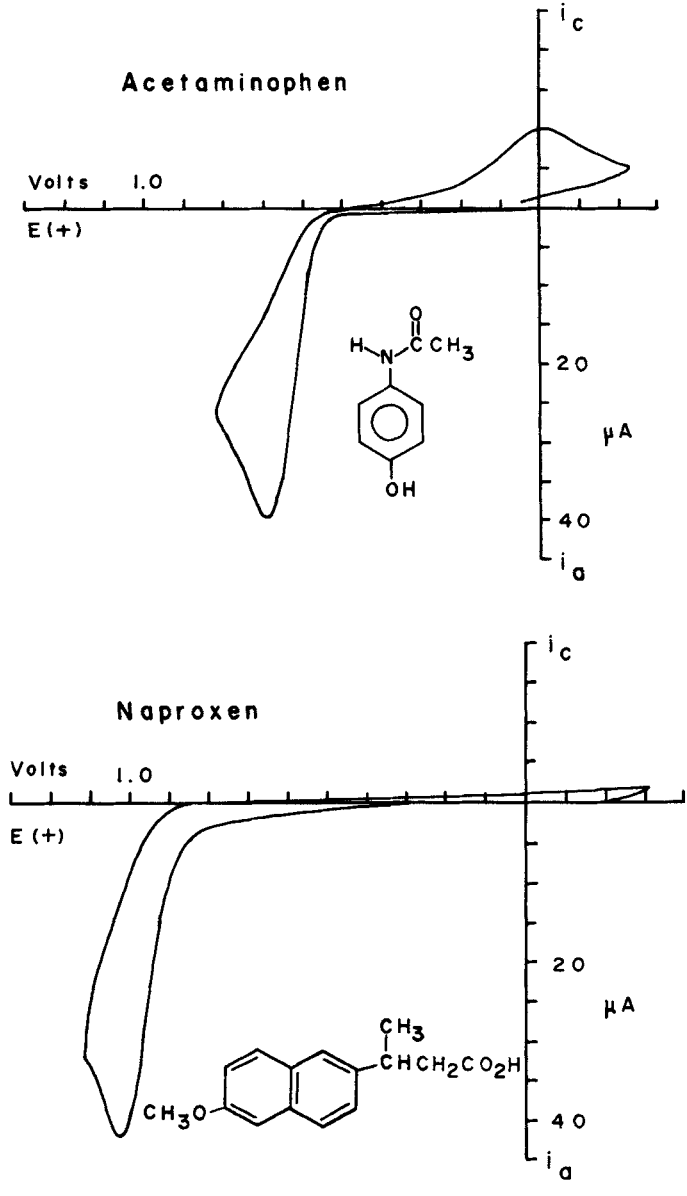


FIGURE 3 (continued)

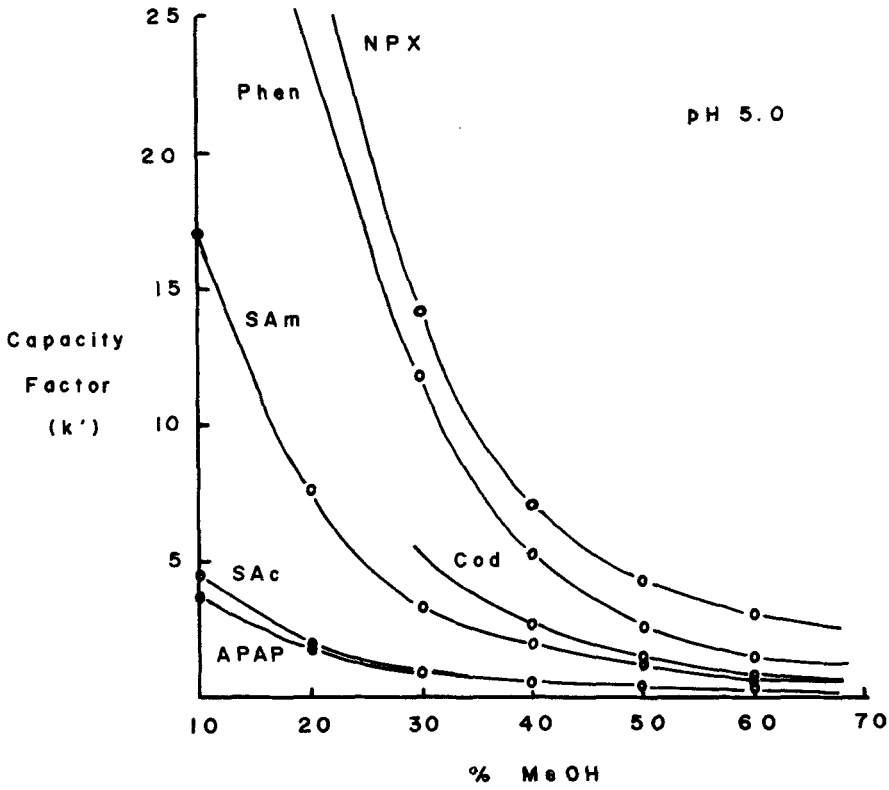


FIGURE 4. Plot of LC capacity factor (k') versus % methanol for several analgesics at pH 5.0.



pounds in Table 1 are listed as useful candidates for additional or alternate internal standards to promote flexibility in applying LCEC to other specific applications. For controlled studies, the electroactive analgesics not under investigation also represent possibile internal standards.

## Serum and Urine Determinations

Serum samples run isocratically as shown in Figure 6 provide reasonable determinations at levels above about 5 μg/mL. NPX and

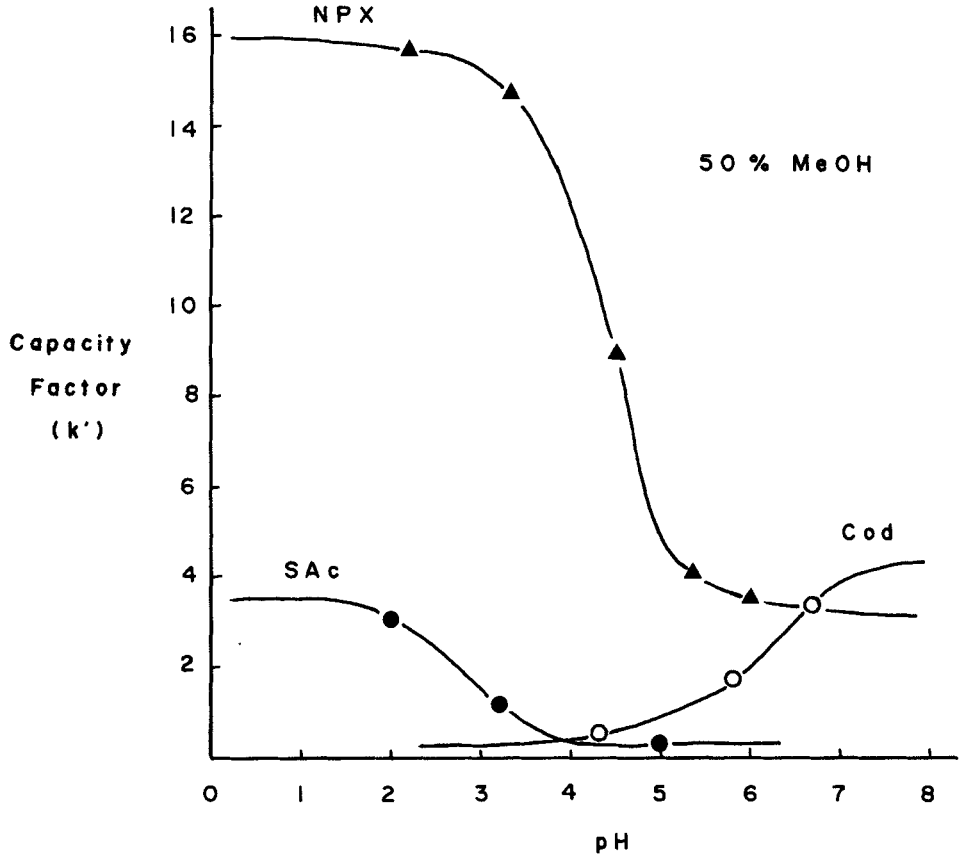


FIGURE 5. Plot of LC capacity factor (k') versus pH for SAc, NPX, and Cod in 50% methanol as mobile phase.

MeS, however, elute rather late as fairly broad peaks, and the interference of endogenous peaks with APAP, SAm, and SAc becomes significant at lower concentration levels. A simple step-gradient elution was evaluated in an attempt to improve resolution of early eluting components and sharpen the peaks of later eluting ones. Inclusion of dual-electrode detection in the "parallel-adjacent" mode enabled simultaneous determination of all analgesics except codeine. Representative chromatograms illustra-

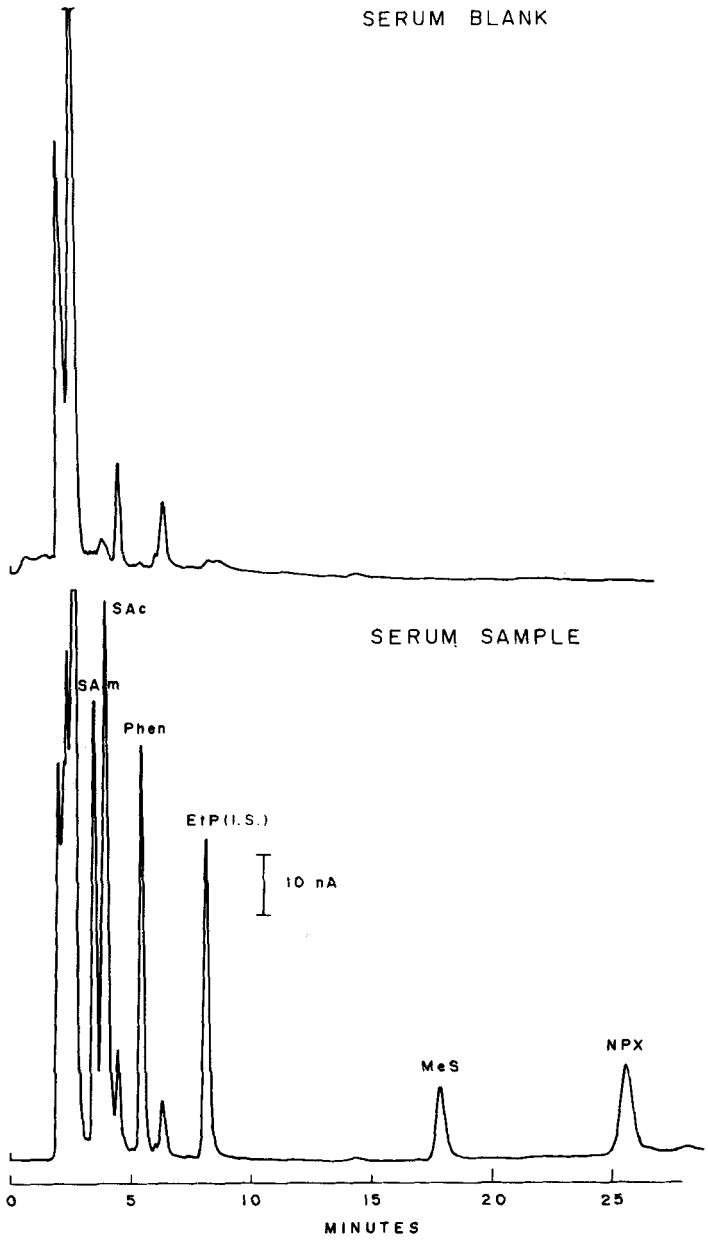


FIGURE 6. Isocratic LC separation of several analgesics in a spiked serum sample. Mobile phase = 0.05 M ammonium MCA (pH 3.2), 0.1 M $NaClO_4$ in MeOH:$H_2O$ (50:50). Detector potential = +1.15 V. Approx. 40 ng of each injected.

ting gradient elution with parallel dual-electrode detection are shown in Figures 7 and 8.

Codeine in serum was determined separately with the 50:50 (v/v) pH 7.0 phosphate:methanol mobile phase. At this pH, Cod is well retained and the retention time of NPX is reduced from 26 to 6 minutes to elute just before Cod. SAm, Phen, NPX, and MeS can be conveniently determined with Cod under these conditions. APAP can often be included in the higher concentration range with adequate selectivity by setting one electrode potential at about +600 mV.

Serum recovery samples at analgesic levels of 2-50 µg/mL were run via step-gradient elution at pH 3.2 and isocratic elution at pH 7.0. Results are listed in Table 2.



One molar phosphoric acid and MCA buffer were added to the injection solution to improve recovery values for SAc and NPX. Initial injections of the supernatant from 50:50 (serum:$CH_3CN$) protein precipitation yielded low recoveries versus aqueous standards. The problem was traced to inadequate pH control in the sample injection solution. Low pH maximizes retention at the head of the column and increases the sharpness of the "plug" injection.

Urine samples contain a fairly high concentration of early eluting endogenous compounds and moderately polar metabolites which generate greater interference in APAP determinations. A simple ethyl acetate extraction proved sufficient to eliminate interferences at the levels evaluated. Note that the working standard must be processed through the extraction step since the analyte and internal standard distribution coefficients are not identical. Although the other analgesics can be readily extracted, SAc and MeS are sufficiently volatile to be partially lost in the evaporation step. The gradient-elution approach proved adequate for determining SAm, SAc, Phen, NPX, and MeS by direct injection as shown in Figure 7. APAP was determined with

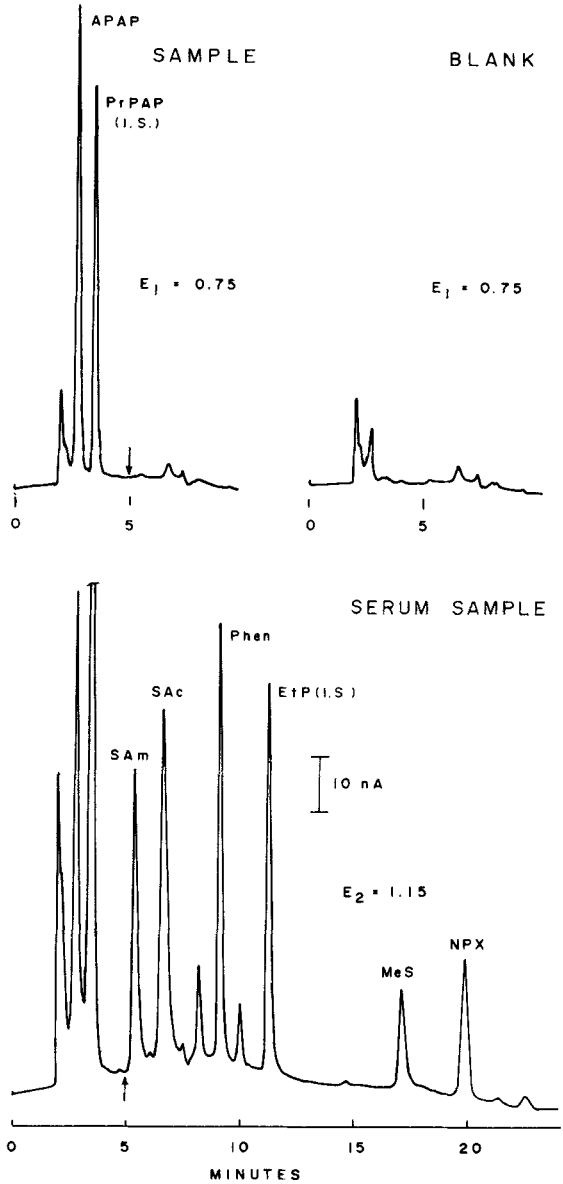


FIGURE 7. Simultaneous determination of several analgesics via step-gradient elution with parallel dual-electrode detection. Mobile phase change from A to B at 5 min. Approx. 40 ng of each injected.

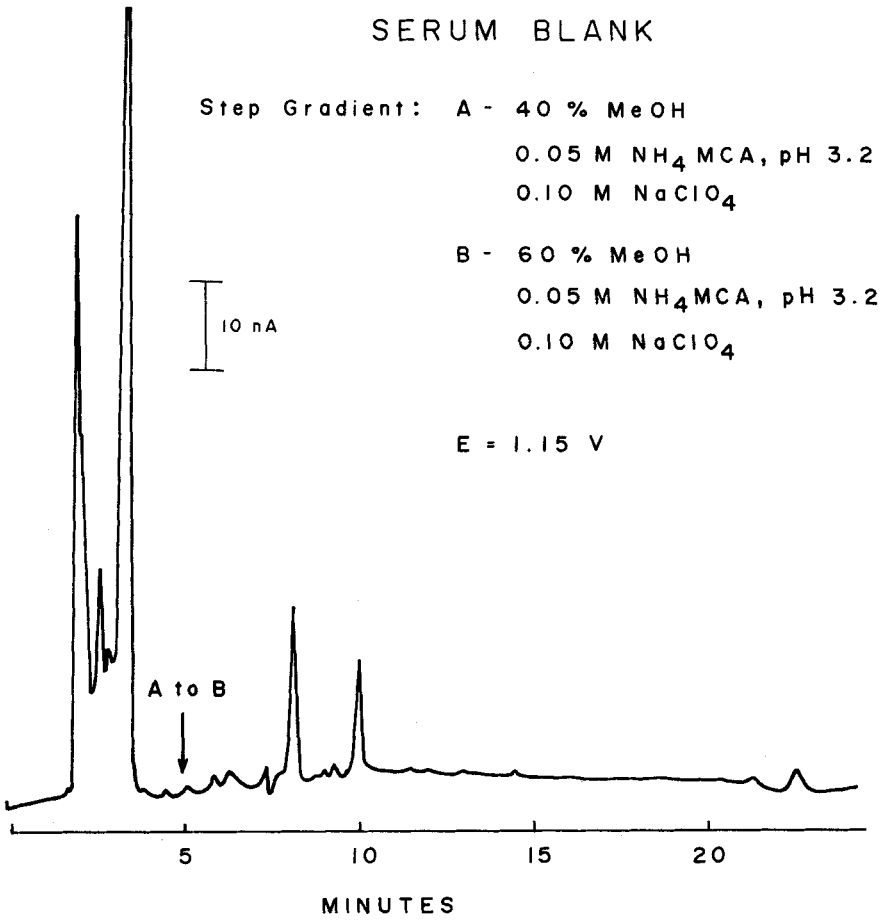


FIGURE 8. Serum blank response for step-gradient elution (A to B) as illustrated in Figure 7.

mobile phase A. Phen, Cod, and NPX could be determined together under isocratic conditions. Urinary recovery results are listed in Tables 3 and 4.

A fabricated $C_{18}$ minicolumn (1.5 cm x 4 mm i.d., 30 µm packing) was evaluated as an alternate sample cleanup tool. After loading a 500 µL urine sample, the column was washed with four

TABLE 2

Serum Recovery Values

| Analgesic | Conc.(µg/mL)[a] | % Rec'y[b] | C.V. |
|---|---|---|---|
| APAP | 2 | 100.2 | 1.0 |
| | 10 | 97.3 | 2.4 |
| | 50 | 98.1 | 0.3 |
| SAm | 2 | 101.7 | 2.0 |
| | 10 | 98.3 | 3.6 |
| | 50 | 102.0 | 3.2 |
| SAc | 2 | 101.7 | 1.4 |
| | 10 | 96.7 | 2.5 |
| | 50 | 99.8 | 5.4 |
| Phen | 2 | 97.3 | 2.4 |
| | 10 | 99.2 | 2.9 |
| | 50 | 98.5 | 1.7 |
| MeS | 2 | 99.0 | 7.4 |
| | 10 | 98.9 | 1.5 |
| | 50 | 100.6 | 1.1 |
| NPX | 2 | 99.0 | 8.9 |
| | 10 | 83.3 | 10.8 |
| | 50 | 87.9 | 9.9 |
| Cod[c] | 2 | 105.0 | 6.3 |
| | 10 | 103.2 | 2.6 |
| | 50 | 104.0 | 7.4 |

a) initial serum concentration
b) n = 4
c) pH 7.0 phosphate, 50% MeOH mobile phase

column volumes (ca. 1.0 mL) of 5% methanol in pH 7.0, 0.1 M phosphate. Four column volumes of 100% methanol were then used to elute and collect the analytes. Endogenous interferences were greatly diminished by this simple procedure to suggest potential value for urinary and other analgesic determinations, especially for analyte pre-concentration where lower level determinations are desired. Unfortunately, the cleanup step did not eliminate an endogenous component which tends to coelute with APAP and exhibit a slightly lower oxidation potential.

TABLE 3

Urine Recovery Values with Step-Gradient Elution

| Analgesic | Conc.(μg/mL)[a] | % Rec'y[b] | C.V. |
|---|---|---|---|
| SAm | 10 | 106.6 | 3.3 |
| | 50 | 100.0 | 1.8 |
| SAc | 10 | 99.8 | 10.0 |
| | 50 | 97.2 | 1.4 |
| Phen | 10 | 112.0 | 2.1 |
| | 50 | 103.8 | 3.5 |
| MeS | 10 | 98.6 | 9.7 |
| | 50 | 102.6 | 1.0 |
| NPX | 10 | 104.4 | 3.4 |
| | 50 | 110.0 | 4.3 |

a) initial urine concentration
b) n = 4

TABLE 4

Urine Recovery Values with Isocratic Elution

| Analgesic | Conc.(μg/mL)[a] | % Rec'y[b] | C.V. |
|---|---|---|---|
| APAP[c] | 5 | 101.7 | 3.1 |
| | 10 | 101.2 | 1.6 |
| | 50 | 95.1 | 0.3 |
| Phen[d] | 5 | 96.9 | 2.8 |
| | 10 | 96.6 | 0.8 |
| | 50 | 100.2 | 0.4 |
| Cod | 5 | 97.9 | 1.3 |
| | 10 | 93.0 | 2.9 |
| | 50 | 97.2 | 2.7 |
| NPX | 5 | 96.7 | 1.9 |
| | 10 | 95.0 | 1.9 |
| | 50 | 99.5 | 0.4 |

a) initial urine concentration
b) n = 4
c) mobile phase A, E = +600 mV
d) mobile phase = 45% methanol/50% 0.2 M phosphate at pH 5.8

Codeine exhibited extensive tailing and peak broadening even on a relatively new column. Lowering the eluent pH to 5.8 and increasing the column temperature to 45°C improved the peak shape somewhat. A representative urine sample under these conditions is shown in Figure 9. The problem of peak integrity proves to be by far the limiting factor in detectability. Codeine exhibits low molar absorptivity at wavelengths of 254 nm and above (19), therefore, work is currently in progress to circumvent this problem and exploit the sensitivity advantage of electrochemical detection.



Gradient Elution

Employment of a stepwise gradient change from 40 to 60 percent methanol was quite successful. Inspection of Figure 7 shows a small "derivative" type baseline disturbance about 2 minutes after the mobile phase change. This is followed by a very slight rise in baseline. Frequently, the baseline disturbance was more pronounced with the baseline rising asymptotically about 10 nA within two minutes to a new plateau. Parameters involved appear to include dielectric constant, pH, temperature, purity of mobile phase and electrode sensitivity. The mobile phase electrolyte concentration was held constant with 0.05 M buffer and 0.10 M $NaClO_4$ to minimize ionic strength changes. A stepwise mobile phase change from pH 3.0 to 4.0 with 0.05 M MCA buffer and 0.1 M $NaClO_4$ in 40% methanol created a baseline shift of similar shape and magnitude. A less abrupt or smooth eluent change with regard to organic modifier and/or pH may provide a quite acceptable baseline transition. Other workers have recently described notable success with linear gradient elution and discussed some of the parameters involved (20-22). At lower electrode potentials the magnitude of gradient-induced baseline shift decreases with the magnitude of the background current. Gradient elution

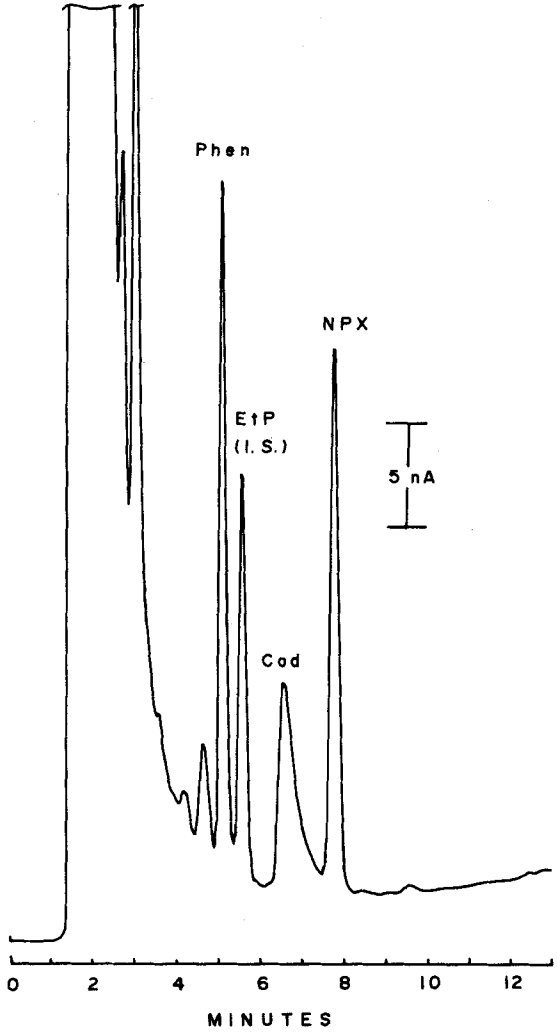


FIGURE 9. Isocratic LC separation of Phen, Cod, and NPX in a spiked urine sample (5 μg/mL). Mobile phase = 0.1 M ammonium phosphate (pH 5.8) in MeOH:$H_2O$ (45:55). Column temp. = 45° C. Detector potential = +1.12 V. Approx. 25 ng of each analyte injected.

is therefore even more amenable to LCEC at lower detector potentials.

It is well known that the mobile phase viscosity changes with methanol content, yielding corresponding changes in frictional heating while eluent is traversing the column. Since background current is temperature dependent, temperature changes will cause observable baseline shifts. The column eluent was thus passed through a section of stainless steel tubing looped in a room temperature water bath to stabilize its temperature. This setup also noticeably decreased random baseline fluctuations, presumably attributed to variable air cooling of the friction-heated eluent. When elevated column temperatures are employed, eluent cooling is extremely effective in reducing total background current.

When the mobile phase is merely altered by organic modifier strength, the EC baseline disturbance has often been observed to be much less than for a 254 nm detector operating at similar sensitivity. Given certain constraints, the application of electrochemical detection to gradient elution separations may frequently equal or surpass the performance of UV detection.



## SUMMARY

LCEC has been shown to provide sensitive determination of several analgesics in complex biological samples with minimal sample preparation. Pertinent chromatographic and detection parameters have beem compiled and discussed to facilitate future applications.

Due to the inherent sensitivity of EC detection for these compounds, practical detection limits depend mostly on the degree of interference from endogenous components. Simple extraction or mini-column cleanup procedures can conveniently eliminate the majority of these interferences to extend useful detection limits.

Baseline stability is another important factor in attaining low detection limits. This can be improved by using isocratic elution or continuous gradients, adequate temperature control, electrochemically pure mobile phases, and uniform mobile phase delivery. The results shown here represent determinations achievable with relatively lowcost equipment.

The analgesics evaluated yield diverse UV absorbance and fluorescence response sensitivities but rather consistent amperometric responses. The detector electrode response appears to be predominately diffusion dependent and hence of very similar magnitude for each analyte.

Parallel dual-electrode detection proved convenient for simultaneous determination of APAP along with compounds of higher oxidation potential. Others have demonstrated the additional utility of this electrode configuration in affirming peak identity by comparing detector response ratios for electrodes at adjacent potentials between sample and standard (23,24). This provides an additional parameter of identification comparable to absorbance ratioing with UV detection.



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