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The Development of an Improved Extraction Method for the Determination of Cocaine Metabolite in Human Urine

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**The Development of an Improved Extraction Method for the
Determination of Cocaine Metabolite in Human Urine**

Chemistry 499

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Advisor: Dr. David Bailey

Chemistry 499 Thesis

Illinois Wesleyan University

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Approval Page

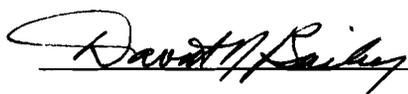
The Development of an Improved Extraction Method for the
Determination of Cocaine Metabolite in Human Urine

by

Leanne M. Nieu Kirk

A paper submitted in partial fulfillment of the requirements for
Chemistry 499 and Research Honors in Chemistry

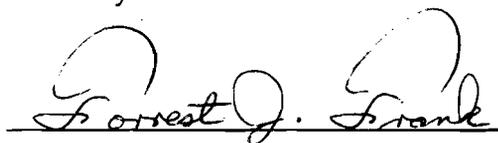
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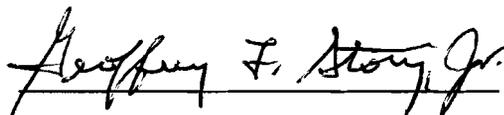
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Abstract

Benzoyl ecgonine (BE) is the primary metabolite of cocaine. Detection of BE in urine is therefore an indication of cocaine usage. The goal of this research is to improve the extraction efficiency in order to increase the sensitivity of the method of determining cocaine usage through urine analysis.

A technique was developed to efficiently extract BE from aqueous solution. Ultraviolet/Visible Spectroscopy was used to determine the extraction conditions for which optimum extraction of BE into organic solvent occurred.

Theory

Due to the prevalence of cocaine abuse in today's society, an efficient, cost-effective method to determine if a person has used cocaine is necessary. Testing methods commonly used are expensive and time consuming, often requiring elaborate sample preparation. The commercially available methods for screening urine for cocaine and its metabolites, such as polarization fluoroimmunoassay, are both expensive and unreliable, often giving false positives due to interferences caused by the presence of other drug metabolites (1). In addition, immunoassays often require sample sizes too large to permit repeated sampling and often do not have enough sensitivity (2).

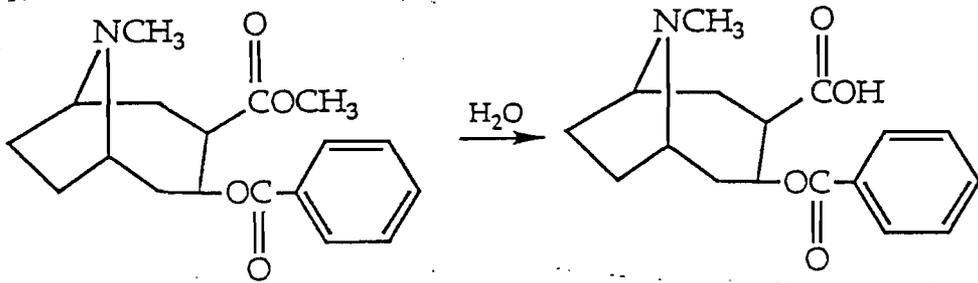
Therefore, a more efficient analysis method is necessary. Urinalysis by High Performance Liquid Chromatography (HPLC) offers several advantages because the sample is easy to obtain; and there is less preparation necessary prior to analysis, which decreases the cost of the method (3). Also, HPLC offers additional advantages of increased reproducibility and greater possibilities of automated analysis (3).

HPLC urinalysis methods are currently being used for the identification and determination of nicotine levels in smokers' urine, by extracting the nicotine present in urine into methylene chloride for analysis (3). Developing an analogous method for cocaine is desirable, thereby allowing for the detection of both tobacco and cocaine use with the same instrument.

Under alkaline conditions, cocaine is metabolized to benzoylecgonine (BE) through the hydrolysis of an ester bond as shown in Figure 1 (6). Benzoylecgonine (BE) is the primary metabolite of cocaine, with 35-45 % of cocaine excreted as BE (1). Detection of benzoylecgonine in urine, therefore, is an indication of cocaine usage. The purpose of this research is to improve

Figure 1

Hydrolysis of Cocaine to Benzoyl ecgonine



the sensitivity of the analytical method for determining cocaine usage. Instrumental techniques can be used to quantify the BE present in a urine sample. Extraction into a non-aqueous solvent is necessary to remove BE from other components in urine that would either interfere in the analysis or harm the HPLC column. Because BE is extremely water soluble, the conditions that give maximum extraction must be found so there is sufficient extraction efficiency, as the range of typical concentration of BE in urine after intranasal use of cocaine is 3-25 ug/L (3).

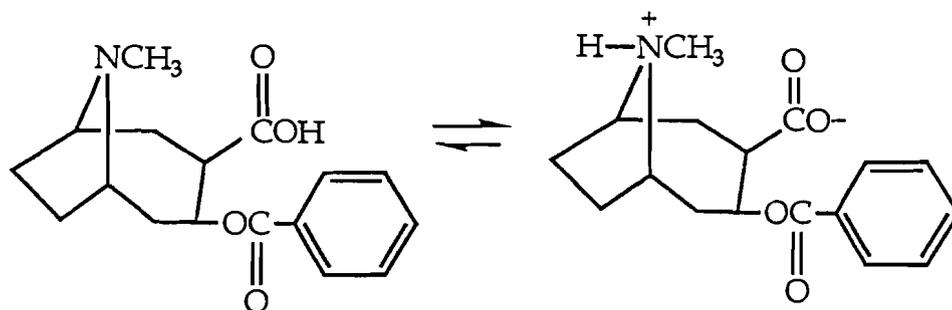
Liquid-liquid extraction is used to isolate a substance dissolved in one solvent by shaking the solution in a separatory funnel with a second solvent that is immiscible with the first. Ideally, the substance of interest is extracted into the second solvent, leaving behind any impurities (4). It is necessary, therefore to find the organic solvent and conditions which most efficiently extract BE from urine.

The underlying principle of extraction is the distribution law, which states in effect that for a dilute solution, a substance is distributed between two immiscible solvents such that the ratio of the concentration in one solvent to the concentration in the second solvent remains constant (4). This ratio is called the partition coefficient. It is approximately the ratio of the solubilities of the solute in each solvent. Three factors control the solubility: the ability of the substance to form hydrogen bonds (that is, its acidity or basicity), its molecular charge, and its molecular dipole moment (4). Because benzoyl ecgonine contains an acidic group and a basic group, it undergoes an intramolecular acid-base reaction to exist in the form of a zwitterion as shown in Figure 2.

A zwitterion acts as an internal salt, exhibiting some of the properties of salts, such as having a large dipole moment, being soluble in water, and

Figure 2.

Benzoyl ecgonine Zwitterion



being amphoteric (5). In a basic solution the zwitterion is deprotonated and exists as an anion as shown in Figure 3. In acidic solution, the zwitterion is protonated and exists as a cation as shown in Figure 4. At some intermediate pH, called the isoelectric point, the two forms must be in equilibrium so that the primary form is the neutral dipolar zwitterion (5).

Extraction efficiency is likely to be pH dependent. The pH of the aqueous BE sample must be adjusted into the proper range for the most efficient extraction. BE has been found to have an isoelectric point between pH 8.1 and 8.8 (3), so it is most likely to be advantageous to perform the extraction of benzoyl ecgonine at this pH.

To select the most effective organic extraction solvent, a variety of factors must be considered. First, the solvent must be immiscible with the sample matrix. While no two solvents are totally immiscible, methylene chloride is almost a water insoluble solvent, having a solubility in water of only 2g/100mL at 20 °C (4). Second, the solvent should have a different density than that of the solution to be extracted; the greater the difference in density between the solutions, the smaller the chance of forming an emulsion between the two solvent layers (6). The density of the organic solvent also determines whether the organic layer will be above or below the aqueous layer. For ease of extraction using a separatory funnel, an organic solvent with a density greater than that of water is preferred. This also has advantages in automating the instrumental method. Finally, a solvent must be chosen which has a sufficiently different retention time than that of benzoyl ecgonine to allow definitive identification of the benzoyl ecgonine peak.

Figure 3.

Benzoyl ecognine in Basic solution

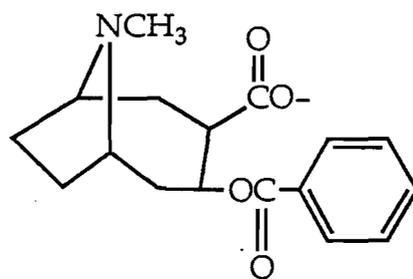
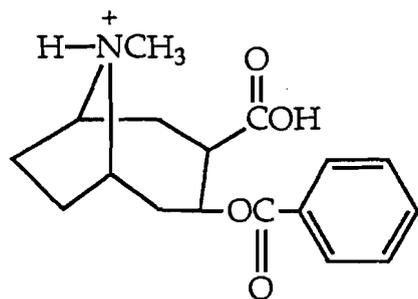


Figure 4.
Benzoyl ecgonine in Acidic Solution



Salting-out is a technique used to increase the efficiency of the extraction of BE from urine. The solubility of amino acids in aqueous solution has been shown to decrease at high salt concentrations (7), therefore it is likely that BE, because it also contains a carboxylic acid and amino group, would have a decreased water solubility by use of this technique. Knowledge of what salt concentration results in maximum extraction efficiency, as well as the effect of the specific cation and anion of the salt, used in conjunction with the other determining factors of extraction previously discussed, will permit determination of the most efficient extraction conditions of BE from urine.

Experimental

Instrumental Conditions

Ultraviolet-Visible Spectrophotometer

Ultraviolet-Visible Spectrophotometry measurements were made on a Perkin-Elmer 559 Ultraviolet-Visible Spectrophotometer.

The Ultraviolet-Visible Spectrophotometer was zeroed using a solvent blank. The cell length was 1 cm. For scanning spectra the wavelength axis was set at 12.5 nm/cm, while the absorbance axis displayed 0-3 Absorbance units.

pH Meter

A Fisher Scientific Accumet pH Meter 900 was used to make pH measurements. The pH meter was standardized using pH 4.00 and pH 10.00 buffer solutions prepared from Meterpak pHDrion buffer tablets.

Procedure

Preparation of Benzoyl Ecgonine from Cocaine Hydrochloride

Cocaine hydrochloride (10 g) was dissolved in a carbonate-bicarbonate buffer, and was then extracted with ether to produce the free cocaine base. The ether was evaporated and the free cocaine collected. The cocaine was then refluxed in 700 mL distilled water for 3 hrs. This solution was then extracted with ether and the aqueous layer evaporated to a small volume. The benzoyl ecgonine was crystallized from this solution by cooling (3). The purity was determined by assay on an Abbott Laboratories TDX instrument.

Preparation of Buffers

Phosphate Buffer

A phosphate buffer was prepared by dissolving 1.695 g KH_2PO_4 (Baker Analyzed Reagent Grade) and 1.765 g Na_2HPO_4 (Baker Analyzed Reagent Grade) in 500 mL distilled water. This yielded a buffer of approximately pH 9.0 (8). To create an approximately 5 M salt solution, 29.516 g NaCl was added to 100 mL of the buffer.

Sodium Borate Buffer

A sodium borate buffer was prepared by dissolving 1.900 g $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$ (Baker Analyzed Reagent Grade) in 500 mL distilled water, yielding a buffer of approximately pH 8.5 (8). To create an approximately 5 M salt solution 29.566 g NaCl was added to 100 mL of the buffer. To create 4, 3, 2, and 1 M NaCl solutions, 2.34, 1.75, 1.17, and 0.58 g NaCl were added to 10 mL aliquots of buffer.

To 10 mL samples of the buffer the following salts were added: 3.44 g KCl, 1.95 g LiCl, 2.69 g NaCl, and 2.47 g NH_4Cl to yield 4.61 M salt solutions; and 0.42 g NaF, 1.03 g NaBr, 0.69 g NaNO_2 , 0.85 g NaNO_3 , and 0.82 g $\text{NaC}_2\text{H}_3\text{O}_2$ to yield 1.0 M salt solutions.

Potassium Phthalate Buffer

A potassium phthalate buffer was prepared by dissolving 1.037 g $\text{KHC}_8\text{O}_4\text{H}_4$ (Baker Analyzed Reagent Grade) in 100 mL of distilled water, yielding a buffer of approximately pH 4.0 (8). To this solution 29.542 g NaCl was added to yield an approximately 5 M salt solution.

Extraction Technique

The general extraction technique used was large scale. The pH of the sample was adjusted by adding 1-15 drops buffer solution to 10.00 mL of 0.4261 g BE/100 mL water sample in a 125 mL separatory funnel. The pH was measured with a pH meter. Two 2.00 mL aliquots of methylene chloride were added. After each addition of solvent, the layers were mixed for one minute by inversion of the separatory funnel. The organic layer was drawn off and retained for UV analysis.

Results and Discussion

After running a series of BE solutions in methanol, the λ_{\max} of BE was determined to be 273.1 nm. The absorbance of BE at this wavelength was used to determine the relative efficiency of extraction performed under different conditions.

For trials one and two, potassium phthalate, potassium phosphate and sodium borate buffers were used to achieve the range of pH values tested. The aqueous solution of BE had an initial pH of approximately 4.8. The pH was increased in .5 pH unit increments, between 4.5 and 9.5 pH units by the addition of buffer. The methylene chloride extracts were then analyzed by UV/Vis. Absorption results of these trials are shown in Tables 1 and 2 and Figure 5. The relative extraction efficiencies did not vary greatly with pH. The differences in efficiencies were thought to be caused by salting-out effects due to the use of different buffers.

To test this theory, the three buffers were made 5 M in sodium chloride. The absorption results of trials three and four, in which the buffers with the added salt concentrations were used, are shown in Tables 3 and 4 and Figure 5. Again, the efficiency did not vary much with pH, however, overall there was an increased extraction efficiency. The highest efficiency appeared to occur between 8.0 and 8.4 pH units, therefore the rest of the extractions were carried out at pH 8.30.

To determine if there might be an effect of the identity of the salt, a series of chloride salts were added to the sodium borate buffer. Potassium chloride had the lowest solubility (34.4 g/100 mL water (8)), therefore the sodium borate buffer was made 4.61 M in each of potassium chloride, sodium chloride, lithium chloride and ammonium chloride. When dissolved, the ammonium chloride solution was strongly acidic, destroying the buffer. The

Table 1

pH vs. Average Relative Extraction Efficiency

Trial 1

<u>pH</u>	<u>R.E.E.</u>
4.74	1.66 ±.03
6.81	1.64 ± .03
7.32	1.60 ± .04
7.83	1.46 ± .03
8.05	1.62 ± .05
8.22	1.61 ± .03

Table 2

pH vs. Average Relative Extraction Efficiency

Trial 2

<u>pH</u>	<u>R.E.E.</u>
4.73	1.87 ± .04
6.41	2.21 ± .03
6.95	1.57 ± .04
7.45	1.54 ± .04
7.70	1.40 ± .05
8.00	1.30 ± .03
8.25	1.65 ± .04
8.50	1.65 ± .03
8.91	1.70 ± .03
9.28	1.62 ± .04

Table 3

pH vs. Average Relative Extraction Efficiencies (Salted Buffers)

Trial 3

<u>pH</u>	<u>R.E.E.</u>
4.86	2.68 ± .03
5.24	2.47 ± .03
2.67	2.50 ± .04
6.23	2.53 ± .03
6.85	2.44 ± .03
7.25	2.53 ± .03
7.64	2.70 ± .04
8.00	2.66 ± .05
8.36	2.97 ± .04

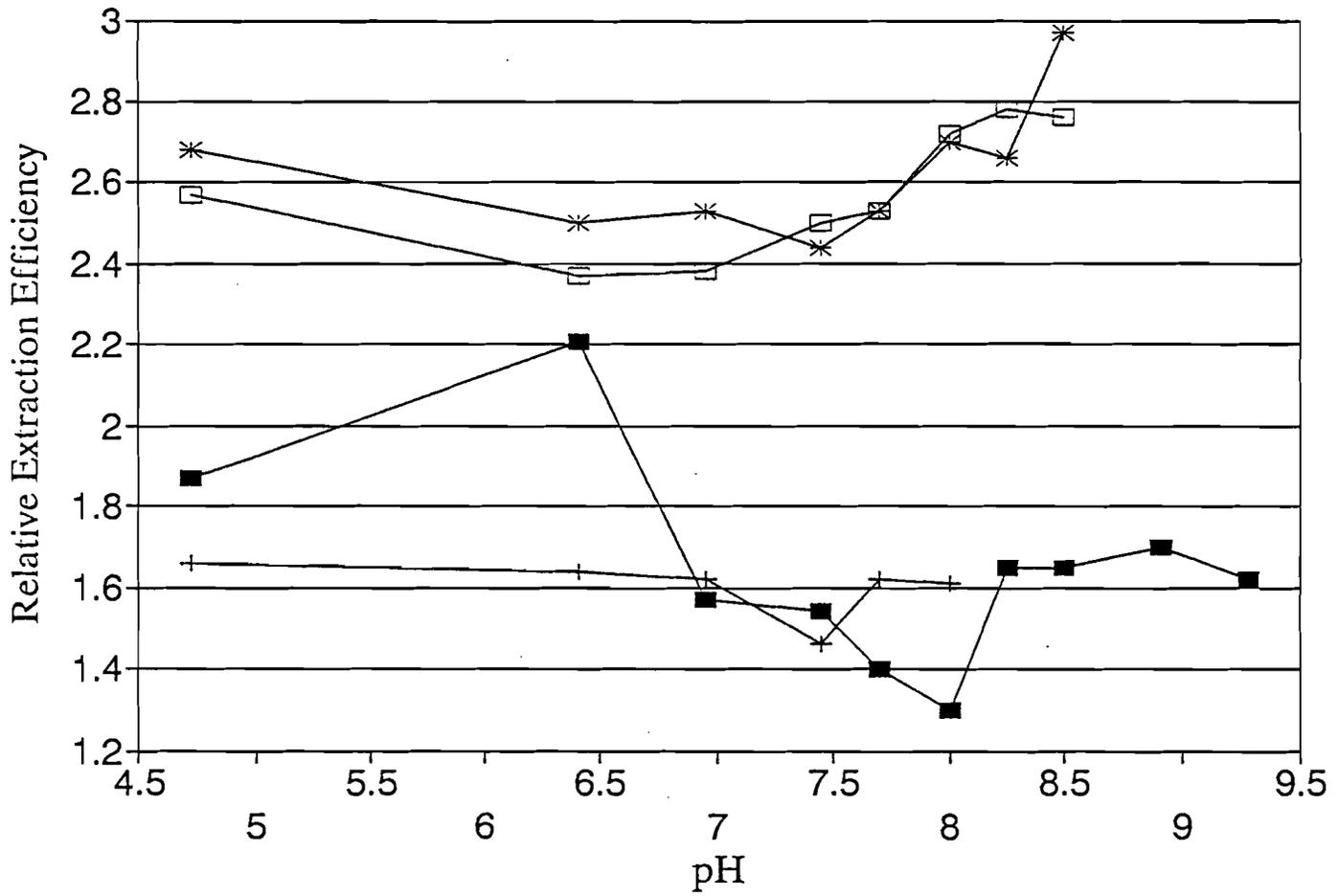
Table 4

pH vs. Average Relative Extraction Efficiencies (Salted Buffers)

Trial 4

<u>pH</u>	<u>R.E.E.</u>
4.86	2.57 ± .04
5.72	2.37 ± .03
6.15	2.38 ± .03
6.67	2.50 ± .04
7.12	2.53 ± .03
7.55	2.72 ± .03
8.00	2.78 ± .03
8.23	2.78 ± .03

Figure 5
Average Relative Extraction Efficiency vs. pH



+ Trial 1 ■ Trial 2 □ Trial 3 * Trial 4

pH of the solution would not increase past 7.10, however, the extraction was still performed. The efficiencies of these extractions did not vary greatly with the cations used (Table 5), but efficiency was slightly increased in the buffer containing potassium chloride.

A series of sodium salts: sodium fluoride, sodium chloride, sodium bromide, sodium nitrite, sodium nitrate, and sodium acetate, were run. Sodium fluoride had the lowest solubility (4.22 g/100 mL water (8)), therefore the sodium borate buffer was made 1.00 M in each salt. The extraction performed using the buffer with sodium chloride added had the lowest efficiency, while the extraction where sodium acetate was used had the highest efficiency (Table 6). The relative efficiency of the sodium acetate extraction was very uncertain because of possible absorption interference from the acetate anion; therefore, it was discarded. The salt which yielded the highest efficiency, then, was sodium nitrate.

A series of extractions was performed to determine the effect of the concentration of the salt. The sodium borate buffer was made to be 1, 2, 3, 4 and 5 M in sodium chloride. There was a general upward trend in the efficiency of the extraction as the salt concentration of the buffer was increased (Table 7).

From the data it seems that there is less dependence of BE extraction efficiency on pH than originally expected. The extraction efficiency is, however, highly dependent on the identity and concentration of salt added to the buffer. The most efficient extractions were performed at high salt concentrations. The cation which yielded the most efficient extraction was potassium, while the anion that yielded the most efficient extraction was nitrate. Since the cation and anion series extractions used different

Table 5

Cation Series Average Relative Extraction Efficiencies

<u>Salt</u>	<u>R.E.E.</u>
NH ₄ Cl	2.56 ± .02
NaCl	2.58 ± .02
LiCl	2.59 ± .02
KCl	2.63 ± .02

Table 6

Anion Series Average Relative Extraction Efficiencies

<u>Salt</u>	<u>R.E.E.</u>
NaCl	2.62 ± .006
NaBr	2.69 ± .04
NaF	2.73 ± .03
NaNO ₂	2.74 ± .02
NaNO ₃	2.77 ± .02
NaC ₂ H ₃ O ₂	2.93 ± .03

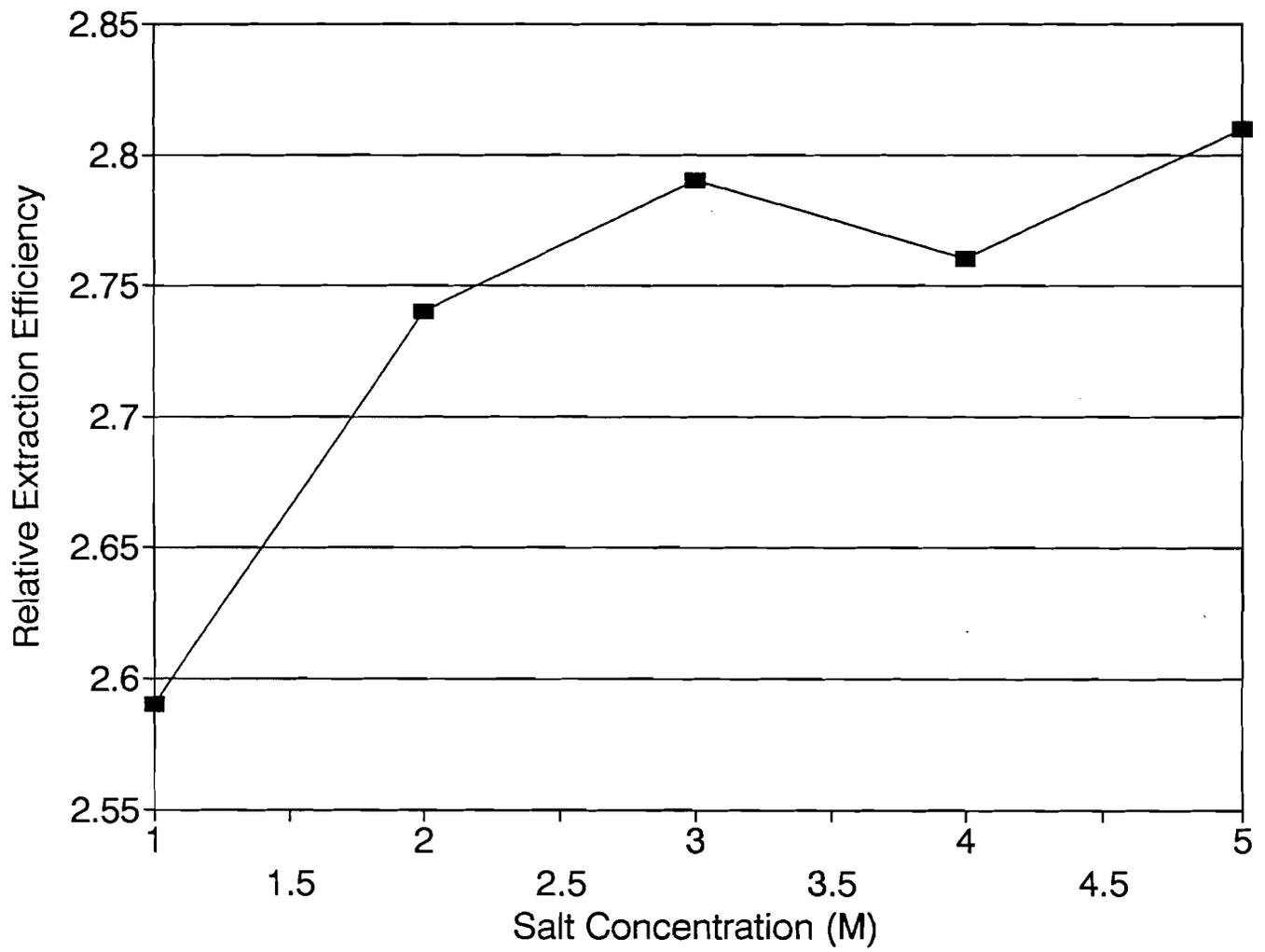
Table 7

Average Relative Extraction Efficiencies vs. Salt Concentration

<u>NaCl Concentration</u>	<u>R.E.E.</u>
1 M	2.59 ± .02
2 M	2.74 ± .03
3 M	2.79 ± .003
4 M	2.76 ± .06
5 M	2.81 ± .01

Figure 6

Average Relative Extraction Efficiency vs. Salt Concentration



concentrations of salt (4.61 and 1.00 M) the two runs can not be completely compared; but it is likely that a buffer that is saturated with potassium nitrate would yield the most efficient extraction of BE. However, the solubility of potassium nitrate in water is 13.3 g/100 mL (8). A saturated solution would only be 1.32 M in potassium nitrate. Because this is a fairly low concentration, the salting-out may not be as great as expected. The effect of using potassium nitrite, with a solubility of 281 g/100 mL water (8), and lithium nitrate, with a solubility of 89.9 g/100 mL water (8), should also be investigated.

During the next phase of research the focus will shift to examine which organic solvent system allows the most efficient extraction. In addition, BE spiked urine samples of varying concentrations will be used to formulate a calibration curve on the HPLC.

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