CAPSULES
preliminary notes and applications from Bioanalytical Systems, Inc.

## *Urinary Vanilmandelic Acid (VMA)*

Vanilmandelic acid (VMA) is the major urinary product resulting from the metabolic degradation of the catecholamines norepinephrine and epinephrine. This pathway is illustrated in Figure 1. In diseases that alter this metabolism, the determination of urinary concentrations of VMA has been shown to be clinically useful. Elevated concentrations of VMA are commonly found in cases of catecholamine secreting tumors such as pheochromocytoma, neuroblastoma, and ganglioneuroma. Determining VMA can differentiate these diseases from those with similar symptoms (e.g. pheochromocytoma versus essential hypertension). Many times these cases are further confirmed by determining plasma or urinary catecholamines, urinary metanephrines, and/or urinary homovanillic acid.

The most commonly used methods for determinimg urinary VMA rely on the spectrophotometric measurement of vanillin following periodate oxidation(1) or on the diazonium compound formed by the reaction of VMA with p-nitroaniline(2). These reactions are not specific for VMA and interferences from other phenolic compounds of endogeneous and dietary origins is common. Gas chromatographic (including GC/MS) methods have been developed but

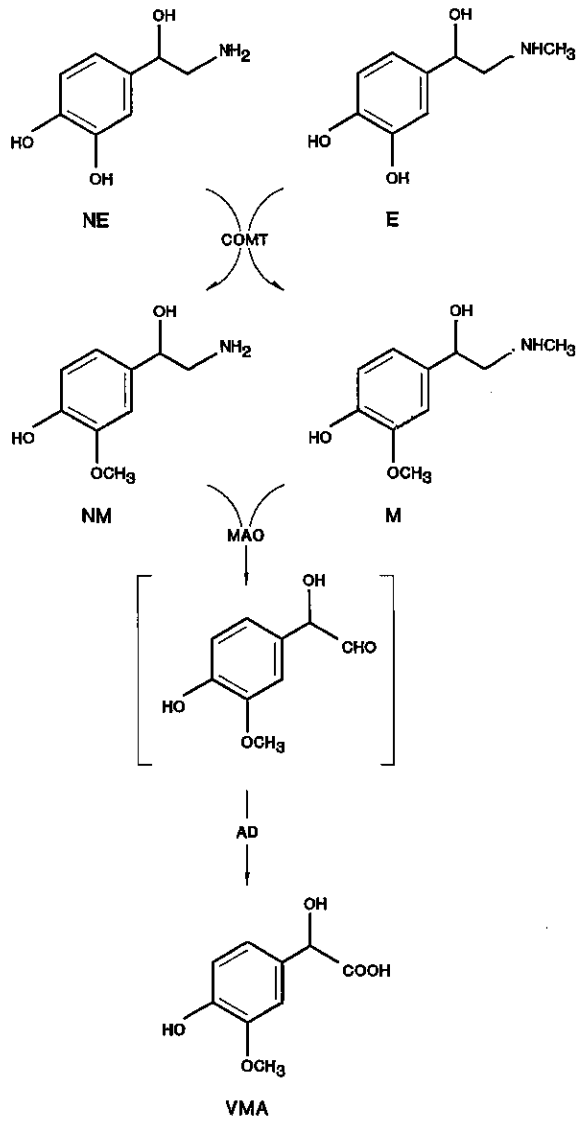


***Figure 1.*** Metabolic pathway of norepinephrine and epinephrine to urinary vanilmandelic acid. NE, norepinephrine; E, epinephrine; NM, normetanephrine; M, metanephrine; VMA, vanilmandelic acid; COMT, catechol-o-methyl transferase; MAO, monoamine oxidase; AD, aldehyde dehydrogenase.

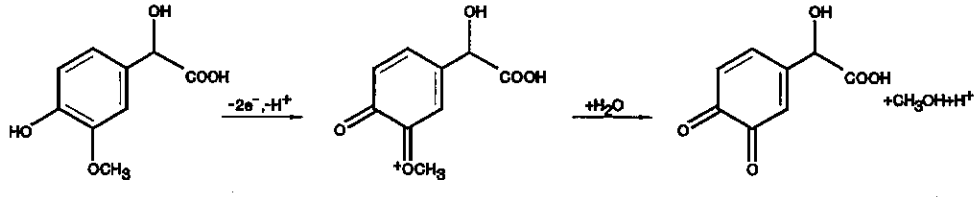


***Figure 2.*** Electrochemical reaction mechanism of VMA in aqueous solution.

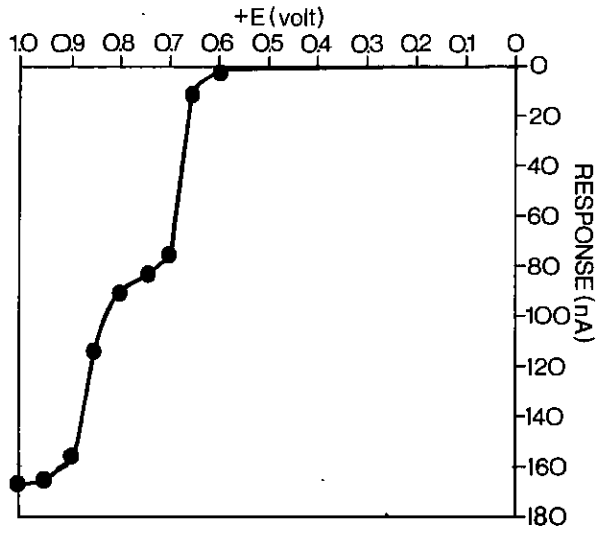


***Figure 3.*** Typical hydrodynamic voltammogram of VMA.

require derivatization and extensive sample clean-up and thus are not applicable for routine use.

LCEC has been used extensively to determine catecholamines and their metabolites from a number of biological samples(3). This Capsule details a procedure for determining urinary VMA concentrations. The method was modified from a published report by Morrisey and Shihabi(4).

**Electrochemistry and LCEC Determination of VMA**

A fundamental requirement for using the LCEC technique is the electrochemical reactivity of the analyte at a suitable electrode. This section reviews this requirement with special emphasis on VMA.

VMA is electrochemically oxidized at a carbon electrode in a aqueous solution according to the reaction mechanism shown in Figure 2. Following the initial two electron transfer at the electrode surface, the resulting oxonium compound undergoes a further chemical reaction yielding an o-quinone product and methanol. This reaction sequence is typical of all compounds containing the vanil moiety (e.g. homovanillic acid, metanephrines, 3-methoxy-4-hydroxyphenylglycol (MHPG). Figure 3 is a voltammogram (the current response as a function of applied potential) of VMA under convective (hydrodynamic) mass transport conditions. The selectivity of a direct electrochemical measurement derives from: (1) not all compounds are electrochemically active, and (2) the ability to screen out certain species by careful choice of applied potential. At a constant applied potential, all compounds that react at this potential will give a response. This electrochemical method can differentiate between compounds that are more difficult to oxidize (or reduce) but not between those that undergo a reaction at lower potentials.

Electrochemical methodologies, then, are inherently low resolution techniques, but they can be very sensitive. This is particularly true in the case of constant applied potential where double layer charging current is negligible and where mass transport to the electrode surface is controlled by hydroynamics. To take advantage of this high sensitivity, the sample must be fractionated before the electrochemical measurement. At present, the most rapid high resolution technique compatible with this electrochemistry is liquid chromatography.

The electrochemical measurement requires that the solution in contact with the electrode have charge or current carrying capacity. This requirement is met by having a solution with a relatively high dielectric constant so that ionic compounds dissolve and dissociate in it. Aqueous buffers, salt solutions, and mixtures of these in polar nonaqueous solvents (e.g., acetonitrile, methanol, THF) meet this criteria. Liquid chromatography experiments that can be performed using mobile phases of this type are generally referred to as "reverse phase" (including ion exchange and ion pair as special cases). The most popular and versatile liquid chromatography being done today uses reverse phase packings with buffered aqueous or aqueous/methanol- acetonitrile mixtures as the mobile phase.

By combining the high separating power of modern reverse phase liquid chromatography with the sensitivity and selectivity of an electrochemical detector, VMA can be easily determined in urinary samples. Very seldom though can a urine sample be injected

directly into the LC column. The exception to this is when the assay involves simple screening for highly elevated concentrations. For monitoring normal or depressed amounts of VMA, some pre-LC sample clean-up is suggested to eliminate chromatographic and electrochemically reactive impurities found in these samples.

Figure 4 illustrates the problems that occur when a urine sample is injected directly on the LC. Interferences make peak quantitation more difficult, reduce sample throughput, and cause rapid electrode passivation. The procedure described below follows a simple double extraction method with final separation and quantitation using LCEC.

## *Materials*

### Reagents

2 M Hydrochloric Acid. Add 83 mL concentrated HCl to ca. 400 mL deionized, distilled water in a 500 mL volumetric flask. Dilute to the mark with deionized, distilled water.

0.10 M, pH 4.0 Phosphate Buffer. Add 3.4 mL concentrated phosphoric acid to ca. 490 mL deionized, distilled water in a beaker. Adjust the pH to 4.0 by using a pH meter, adding either a concentrated solution of sodium hydroxide or solid pellets while simultaneously stirring the solution. Transfer the solution to a 500 mL volumetric flask and dilute to the mark with deionized, distilled water. *This solution should be made fresh every day.*

Ethyl Acetate. Analytical Reagent Grade (ACS or equivalent).

0.20 M, pH 3.0 Monochloroacetate (MCAA) Buffer with 2 mM Ethylenediaminetetraacetate (EDTA). Dissolve 37.7 g monochloroacetic acid, 12.5 g sodium hydroxide, and 1.5 g EDTA (disodium salt) in approximately 2 L of deionized, distilled water. Adjust the pH to 3.0 using a pH meter, adding solid MCAA or NaOH as needed while stirring. This is the chromatographic mobile phase and should be filtered (0.2 µm pore size membrane filter) and degassed (stirring while under aspirator vacuum for about 10 minutes) prior to use.

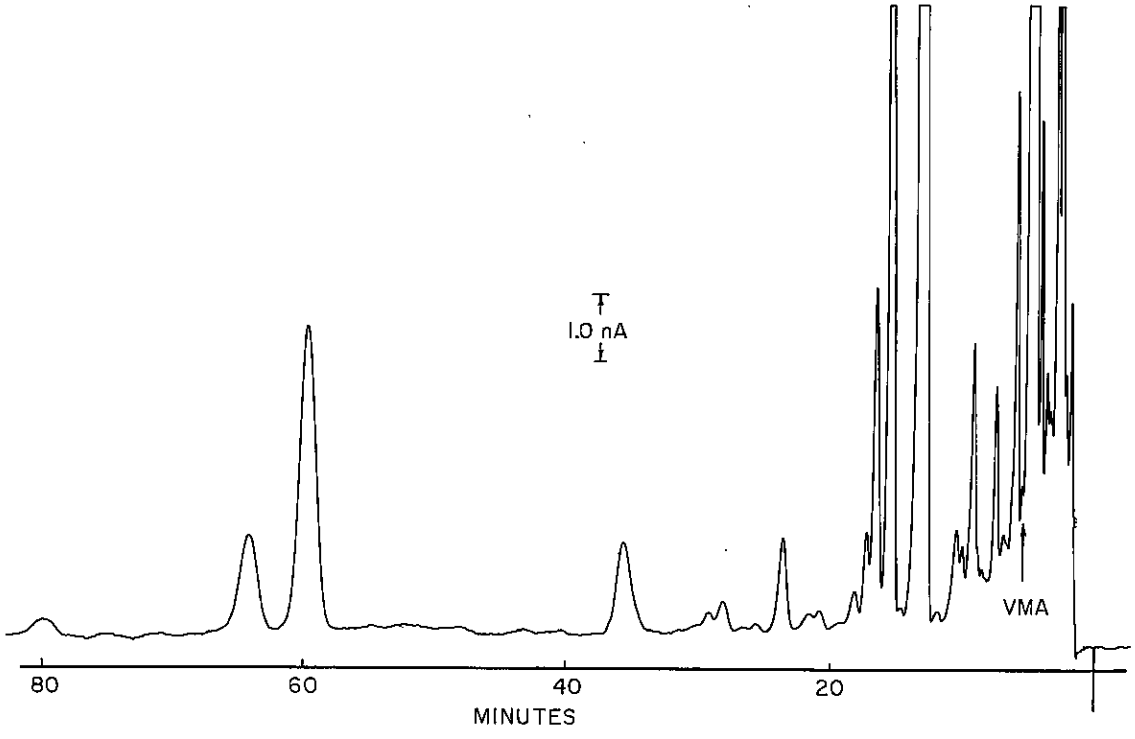


***Figure 4.*** Typical chromatogram following 50-fold dilution of the urine sample and direct injection into the LCEC system. Chromatographic conditions as described in Experimental.

VMA Stock Solution. 1.2 mg VMA/mL in 0.1 M perchloric acid. Dissolve 60 mg VMA standard (P/N CF-1030, Bioanalytical Systems) in 50 mL of 0.1 M perchloric acid.

Synthetic Sample. 0.10 M, pH 4.0 phosphate buffer containing 6 µg VMA/mL. Prepare phosphate buffer as described above. Pleace exactly 0.50 mL VMA stock solution in 100 mL volumetric flask and dilute to mark with phosphate buffer.

**Recommended Accessories**

Clin Elut Extraction Columns. Model CE 1001-M, 0.3 mL total capacity.

Centrifuge tubes. 12-15 mL capacity, conical, glass or polypropylene.

Small Centrifuge. Model MF-C (P/N MF 5060, Bioanalytical Systems).

Volumetric or Mechanical Pipets. 3 mL, 125 microliters, 5 mL sizes.

Mobile Phase Filtering Assembly. BAS P/N MF-6126, an all glass system which includes regenerated cellulose membranes (0.2 µm pore, 47 mm diameter). A 500 mL suction flask is used as an inline trap. Use 0.2 µm membrane filters (P/N MF-5620, regenerated cellulose or MF-5621, Nylon-66. Both are 0.2 µm pore size).

## *Experimental Conditions*

Liquid Chromatograph: LC-154T (Bioanalytical Systems), BAS 200, BAS 460 or BAS 400
Mobile Phase: 0.20 M, pH 3.0 monochloroacetate with 2 mM EDTA
Flowrate: 1.5 mL/min
Stationary Phase: Biophase ODS 5 µ (P/N 6017, Bioanalytical Systems)
Temperature: 30°C
Amount Injected: 100 µL
Detector: Glassy carbon working electrode (Bioanalytical Systems)
Detector Potential: +0.75 volts (vs. Ag/AgCl)
Controller Sensitivity: 10 or 20 nA/V

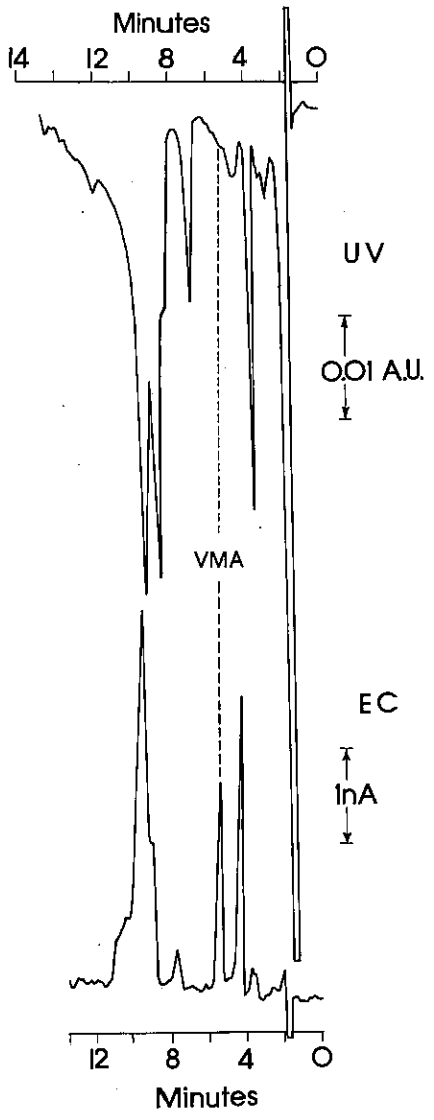


***Figure 5.*** Comparison of UV (254 nm, upper trace) and EC (lower trace) detection. Chromatographic conditions and sample workup procedure are the same as described under Experimental Conditions.

**Procedure**

1. Place Clin Elut columns in an appropriate rack with 15 mL glass centrifuge tubes positioned below each column.

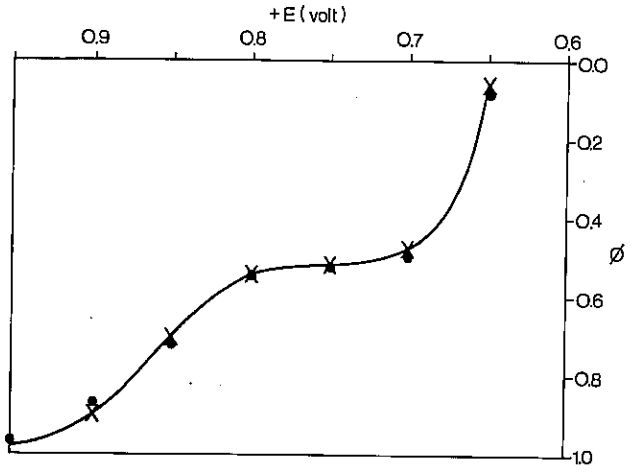


***Figure 6.*** Comparison of normalized hydrodynamic voltammograms of VMA from a urine sample (•) and a standard solution (x). Chromatographic conditions and sample work-up procedure are the same as described under Experimental.

2. Add 5 mL of 0.1 M, pH 4.0 phosphate buffer solution to each glass collection tube.

3. Add 125 µL of 2 M HCl solution to each column.

4. Add 125 µL of urine sample or synthetic sample solution to each column. Wait approximately 3 minutes. At least two synthetic samples should be assayed with each batch of urine samples.

5. Add 3 mL of ethyl acetate to each Clin Elut column. Wait approximately 5 minutes for complete elution.

6. Cap and vortex mix each collection tube for a least 2 minutes.

7. Centrifuge each collection tube at least 5 minutes.

8. Inject 100 µL of the lower aqueous phase into the LC.

**Sample Calculations**

The amount of VMA in each urine sample is determined by comparison with a synthetic sample with a known amount of VMA carried through the same procedure. At least two synthetic samples should be run with each batch of 12-15 urine samples. The concentration of VMA in the unknown sample can be determined from the following equation:

$$[VMA]_{unknown} \quad (\mu g/mL) =$$

$$(\text{peak height, VMA})_{unknown} \quad X$$

$$\frac{[VMA]_{synthetic} \ (\mu g/mL)}{(\text{peak height, VMA})_{synthetic}}$$

**Example:** A synthetic sample containing 6 µg VMA/mL gave a peak height of 10.0 nAmps. The unknown urine sample gave a peak height of 3.6 nAmps. The VMA concentration in the urine sample is:

$$[VMA]_{unknown} = 3.6 \text{ nAmps} \times \frac{6.0 \ \mu g/mL}{10.0 \text{ nAmps}} = 2.2 \ \mu g/mL$$

Often the VMA concentration is expressed as µg/mg creatinine or mg/24 hour. The amount of creatinine per mL of urine is determined separately. To obtain µg VMA/mg creatinine, simply divide the amount of VMA/mL as determined above by the amount of creatinine in mg/mL. Expressed as an equation, this becomes:

$$[VMA] \ (\mu g/mg \text{ creatine}) = \frac{[VMA] \ (\mu g/mL)}{[\text{creatinine}] \ (mg/mL)}$$

To express the concentration as mg/24 hours, multiply the VMA concentration determined above by the total volume of urine collected in the 24 hour period and divide by 1000 to convert µg to mg. The conversion equation is:

$$[VMA] \ (mg/24 \text{ hr.}) = [VMA] \ (\mu g/mL) \times (\text{total Urine Volume, mL}) \times 1 \text{ mg}/1000 \ \mu g$$

**Results and Discussion**

The urinary VMA procedure described has been evaluated for routine clinical use. A rapid sample preparation was employed to avoid long chromatographic analysis times. Qualitative analysis was performed to assure adequate chromatographic resolution and purity. The method was used to determine VMA concentrations from healthy urine samples and diseased patients. From these determinations, absolute and relative recoveries were calculated, the precision of the assay evaluated, and detection limits ascertained.

The method described in this note was modified from a previously published report(4). In the above procedure, a slightly more elaborate pre-LC isolation procedure based on a commercially available liquid-liquid extraction column, the Clin Elut tube, is employed, rather than using the simple phase separation technique of reference 4. For some urine samples, extraneous compounds were found that either interfered with the VMA peak or had excessive retention times when the simple extraction was used. These were not found using the Clin Elut column extraction. Both the reliability and the analysis time per sample were reduced. In addition, chromatographic resolution and minimum quantifiable amounts were improved because of the higher efficiency Biophase ODS column used in this work. The detection limits from a urine sample at a signal/noise ratio of 3 was 0.2 µg/mL, which corresponds to 500 pg per 100 µL injected, at a 100 pAmp noise level. The absolute recovery was 86 ±3% r.s.d. (N = 5) at a concentration of 2.5 µg/mL. The relative recovery averaged 99 ±3%. The method was shown to be linear from 2-20 µg/mL in urine samples collected from laboratory personnel. Approximately 30 samples can be assayed per day including sample work up and chromatographic run time.

A typical chromatogram illustrating the separation of VMA in a urine sample is shown in the lower trace of Figure 5. The upper trace is a chromatogram of the same urine sample using a UV (254 nm) detector in series with the electrochemical detector. For the VMA determination, clearly the electrochemical detector exhibits superior selectivity and sensitivity.

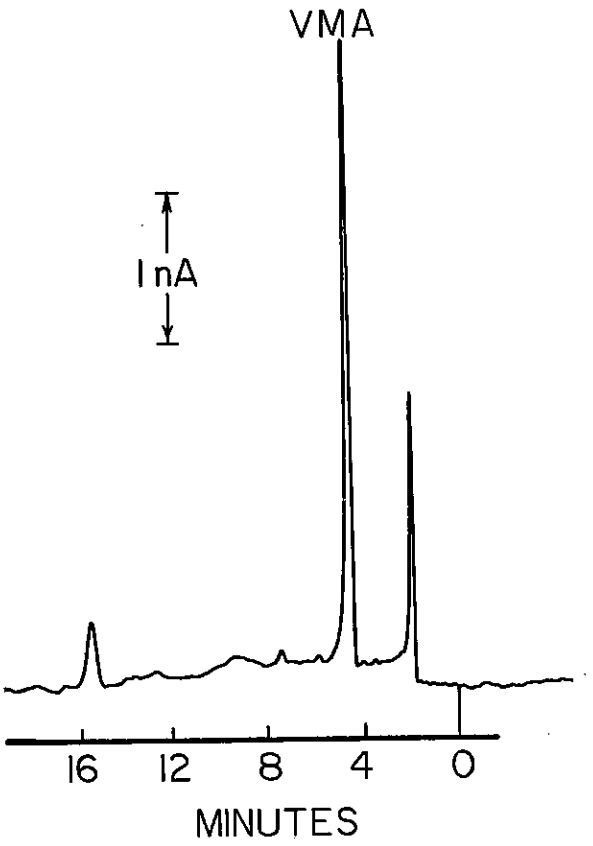


***Figure 7.*** Typical chromatogram illustrating the "dilute and inject" procedure for screening urinary samples for elevated amounts of VMA. Neuroblastoma urine sample diluted 1:1000 with mobile phase and injected onto the LC. The VMA response corresponds to 7.0 ng injected or 70 ug VMA/mL urine. Chromatographic conditions are the same as described in Experimental.

Figure 6 is a comparison of normalized hydrodynamic voltammograms (HDV's) of a synthetic VMA sample versus the VMA peak in the actual urine. The overlap of these curves confirms the purity of the VMA peak in the sample and demonstrates sufficient resolution. Coeluting interferences would show up as deviations in the sample plot as compared to the authentic VMA plot.

Screening urine samples for elevated concentrations of VMA was accomplished by simply diluting the sample and injecting. Figure 7 illustrates a

chromatogram of a urine sample from a neuroblastoma patient following a 1000-fold dilution with mobile phase. Interferences may occur since no sample clean-up is involved. All questionable sample must be assayed according to the extended proceedure to accurately determine the amounts present.

For urinary VMA determinations, liquid chromatography with electrochemical detection is a selective approach with more than adequate sensitivity and detection limits. When combined with simple extraction steps, the technique is applicable to assaying large numbers of samples in a minimal amount of time with the required precision and accuracy.

**CAUTION**

Liquid chromatography with electrochemical detection is a highly useful tool for biomedical research. As with any instrumental technique, the precision and accuracy of the measurement depends on the instrumentation, the skill and knowledge of the operator and the integrity of the sample preparation procedure. Use of these techniques for medical diagnosis and accountability for the same rests entirely with the user of this equipment.

**References**


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*2. S. E. Gitlow, L. M. Bertani, and A. Rausen, Cancer (Philadelphia), 25 (1970) 1377.*

*3. "Recent Reports on Liquid Chromatography with Electrochemical Detection," R. E. Shoup, Ed., Bioanalytical Systems Inc., W. Lafayette, IN, 1981.*

*4. J. L. Morrisey, and Z. K. Shihabi, Clin. Chem., 25 (1979) 2043.*