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Determination of oxalate in urine and plasma using reversed-phase ion-pair high-performance liquid chromatography with tris(2,2′-bipyridyl)ruthenium(II)-electrogenerated chemiluminescence detection

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Abstract

Oxalate is quantitated in both urine and plasma samples using reversed-phase ion-pair high-performance liquid chromatography (HPLC) with tris(2,2′-bipyridyl)ruthenium(II) [$Ru(bpy)_3^{2+}$]-electrogenerated chemiluminescent (ECL) detection. Underivatized oxalate was separated on a reversed-phase column (Zorbax ODS) using a mobile phase of 10% methanol in 100 m*M* phosphate buffer at pH 7.0. The eluted compounds were combined with a stream of 2 m*M* $Ru(bpy)_3^{2+}$ at a mixing tee before the ECL flow-cell. In the flow-cell, $Ru(bpy)_3^{2+}$ is oxidized to $Ru(bpy)_3^{3+}$ at a platinum electrode, and reacts with oxalate to produce chemiluminescence (CL). Urine samples were filtered and diluted prior to injection. Plasma samples were deproteinized before injection. A 25-μl aliquot of sample was injected for analysis. Possible interferants, including amino acids and indole-based compounds, present in biological samples were investigated. Without the separation, amino acids interfere by increasing the total observed CL intensity; this is expected because they give rise to CL emission on their own in reaction with $Ru(bpy)_3^{3+}$. Indole compounds exhibit a unique interference by decreasing the CL signal when present with oxalate. Indoles inhibit their own CL emission at high concentrations. By use of the indicated HPLC separation, oxalate was adequately separated from both types of interferants, which thus had no effect on the oxalate signal. Urine samples were assayed by both HPLC and enzymatic tests, the two techniques giving similar results, differing only by 1%. Detection limits were determined to be below 1 μM (1 nmol/ml) or 25 pmol injected. The working curve for oxalate was linear throughout the entire clinical range in both urine and plasma.

1. Introduction

Knowledge of the oxalate concentration in blood and urine is important in the investigation of many serious clinical disorders, such as chronic renal failure [1], urinary stone disease [2], primary hyperoxaluria, and intestinal malabsorption [3]. If these conditions are recognized early, as indicated by blood or urine oxalate concentrations, complications may be avoided [4]. Because of this, accurate measurement of oxalate in biological samples has gained importance in the clinical laboratory.

Many methods have been described for the determination of oxalate in biological fluids. These include precipitation of oxalate as the

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calcium salt, enzymatic techniques, isotope dilution, gas chromatography and ion chromatography, among others [2,3,5–10]. Many of these techniques suffer from lack of sensitivity, poor recovery due to extensive sample handling, radioisotope use, limited dynamic range, long incubation times, extensive sample preparation, and inaccurate results. Many methods are not suitable to detect oxalate in both urine and blood. Several high-performance liquid chromatographic (HPLC) methods require pre-column derivatization of oxalate or extensive sample preparation and clean-up, and are not acceptable for routine clinical analysis [11,12].

Reversed-phase ion-pair HPLC has been shown to be suitable for separation of oxalate in biological samples [2,10,13–16]. Detection schemes include UV absorbance at 220 nm [13], direct amperometric determination of oxalate [15,16], amperometric detection using complexation at a copper electrode [14], and amperometric detection following reaction with oxalate oxidase [10]. UV absorbance suffers from lack of sensitivity, and is therefore useful only for urine samples. Of the amperometric approaches, the combination with oxalate oxidase did not have a sufficiently low detection limit to detect oxalate in the plasma clinical range, detection with a copper electrode has not been applied to plasma samples, and direct amperometry of oxalate has generally shown poor correlation with commercial enzymatic test kits.

Chemiluminescence (CL) has become an attractive detection method for liquid chromatography due its very low detection limits and wide linear working ranges, while using relatively simple instrumentation [24]. Tris(2,2′-bipyridyl)ruthenium(II) ($Ru(bpy)_3^{2+}$) chemiluminescence has been shown to be a valuable technique for the determination of oxalate [17,18]. $Ru(bpy)_3^{2+}$ chemiluminescence has also been applied to the determination of aliphatic and alicyclic amines [19,20], amino acids [21,22], NADH [18,23], and recently for the HPLC separation of dansyl derivatized amino acids [25]. The oxidation–reduction reaction mechanism for $Ru(bpy)_3^{2+}$ chemiluminescence has been postulated by Rubinstein et al. [17].

$$Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{3+} + e^-$$

$$Ru(bpy)_3^{3+} + \text{reductant} \rightarrow \text{product} + [Ru(bpy)_3^{2+}]^*$$

$$[Ru(bpy)_3^{2+}]^* \rightarrow Ru(bpy)_3^{2+} + h\nu$$

In the present case, oxalate is the reductant and CO_2 is the product [18]. $Ru(bpy)_3^{2+}$ is oxidized to $Ru(bpy)_3^{3+}$ at an electrode, and the electrogenerated chemiluminescence (ECL) intensity measured is directly proportional to the amount of reductant present.

In this paper, we report a new detection technique for the quantitative determination of oxalate in urine and plasma by using reversed-phase ion-pair high-performance liquid chromatography with post column reaction detection based on electrogenerated chemiluminescence with $Ru(bpy)_3^{2+}$. Possible interferants present in biological samples are also investigated using flow injection analysis (FIA). The method is demonstrated with both urine and plasma samples. The results from urine samples using this method are compared to results from a commercial enzymatic test kit for determination of oxalate in urine.

2. Experimental

2.1. Instrumentation

For characterizing the interferants and their interaction with oxalate, a flow-injection analysis setup was used which is shown in Fig. 1. A Rainin Rabbit peristaltic pump (Woburn, MA, USA) was used to deliver the flowing buffer stream through a Valco Model E60 injector (Houston, TX, USA) with a 70-μl injection loop. The tubing I.D. was 0.31″ (0.8 mm). The buffer used for the FIA experiments was 50 m*M* acetate at pH 5.0 with 200 m*M* KNO_3 as supporting electrolyte. This buffer has been shown to work well with the $Ru(bpy)_3^{2+}$ ECL system [25]. The samples and the CL reagent [$Ru(bpy)_3^{2+}$] were mixed and then injected to-

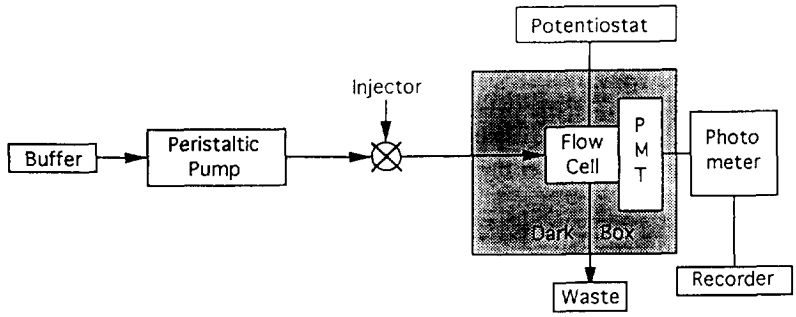

Fig. 1. Schematic diagram for chemiluminescence detection in flow-injection analysis setup used for study of interferants.

gether. The ECL emission was initiated in an electrochemical flow-cell contained in a "dark box".

The electrochemical/chemiluminescence (ECL) flow-cell, shown in Fig. 2, was constructed from an LC-electrochemical detection dual platinum electrode (Bioanalytical Systems, West Lafayette, IN, USA) and a plexiglas window for detecting the CL emission. A 127-μm Mylar spacer is placed between the electrode block and the window to create a cell volume of approximately 9.2 μl. The plexiglas window of the flow-cell was mounted directly across from a photomultiplier tube (PMT) (RCA 1P28A, Lancaster, PA, USA). The PMT was biased at 900 V using a Pacific Instruments Model 227 high-voltage power supply (Concord, CA, USA). Current from the PMT was measured using a Pacific Instruments Model 126 photometer and a Houston Instruments Model B5117 strip chart recorder (Austin, TX, USA). The working electrodes

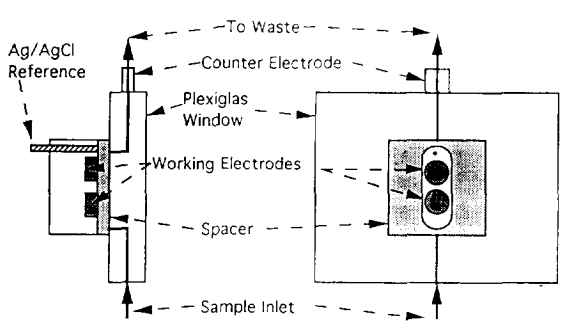

Fig. 2. Schematic diagram for ECL flow-cell used in both FIA and HPLC setups.

in the flow-cell were held at a potential of +1.30 V vs. Ag/AgCl reference electrode to convert $Ru(bpy)_3^{2+}$ to $Ru(bpy)_3^{3+}$. The potential for these experiments was applied using a Bioanalytical Systems BAS 100A electrochemical analyzer. A Bioanalytical Systems screw-in Ag/AgCl reference electrode and a stainless steel counter electrode at the cell exit were used. $Ru(bpy)_3^{2+}$ has previously been immobilized on the working electrode surface in a Nafion ion-exchange polymer [18], but here all experiments are done using solution-phase $Ru(bpy)_3^{2+}$ CL.

For HPLC determination of oxalate, the FIA instrumental setup was modified as shown in Fig. 3. An Altex Model 110A HPLC pump (Berkeley, CA, USA) was used to deliver the mobile phase through 0.01" HPLC tubing to retain resolution. The HPLC system consisted of a silica pre-column, a Partisil ODS guard-column (70 × 2.0 mm I.D.) and a DuPont Zorbax ODS reversed-phase column (5 μm, 250 × 4.6 mm I.D.). A 25-μl injection loop was used. The mobile phase was phosphate buffer–methanol (90:10, v/v), and was delivered at a flow-rate of 1.0 ml/min. The column conditioning solution consisted of methanol–water (25:75, v/v) with 2 m*M* cetyltrimethylammonium bromide. After the analytical column, the effluent was combined with a solution of 2 m*M* $Ru(bpy)_3^{2+}$ at a mixing tee contained in the dark box. The $Ru(bpy)_3^{2+}$ solution was delivered by a Rainin Rabbit peristaltic pump through a check valve at a flow-rate of 0.25 ml/min. The column effluent and CL reagent streams mix directly before the flow-cell in the dark box. The flow-cell and detection

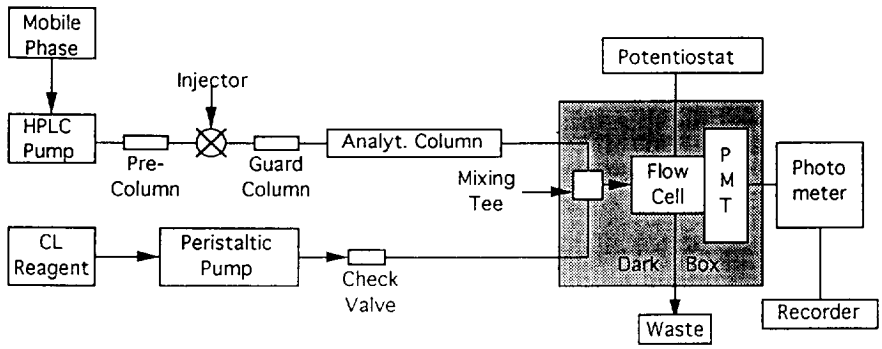

Fig. 3. Schematic diagram for HPLC system with chemiluminescence detection.

instrumentation was the same as that described for the FIA experiments.

2.2. Reagents

3-Indoxyl sulfate, indole-3-acetic acid, indole, 5-hydroxy-l-tryptophan, and 5-hydroxytryptamine were purchased from Sigma (St. Louis, MO, USA). Tris(2,2′-bipyridyl)ruthenium(II) chloride hexahydrate and cetyltrimethylammonium bromide were purchased from Aldrich (Milwaukee, WI, USA). Also, for comparison, a commercial oxalate test kit was purchased from Sigma Diagnostics. The remaining samples were prepared from reagent-grade or better chemicals purchased from commercial sources. For all solutions, water was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA). Methanol for the mobile phase was HPLC grade. The mobile phase was a phosphate buffer adjusted to pH 7.0 with 1 *M* HCl, with 10% methanol (v/v). The mobile phase was degassed by bubbling with nitrogen prior to HPLC analysis. Blood samples were obtained from the Champaign County Blood Bank. These blood samples had been collected with an anticoagulant which consisted of a citrate, phosphate, dextrose, and adenine solution (Baxter Healthcare Corp., Deerfield, IL, USA). Samples for standard addition were made from a freshly prepared stock solution of 5 m*M* oxalate.

2.3. Evaluation of interferants by FIA

Several compounds present in biological samples that pose as potential interferants when determining oxalate using $Ru(bpy)_3^{2+}$ chemiluminescence have been reported [18]. These primarily include free amino acids and amines. Several of the more important compounds and interferants are listed in Table 1, with their corresponding concentration ranges in urine and plasma [26]. The CL of these compounds and their interaction with oxalate were studied using flow-injection analysis (FIA). All samples injected contained the compound(s) of interest at various concentrations and $Ru(bpy)_3^{2+}$ at a concentration of 500 μM. A 70-μl volume of sample was injected into a flow stream of 100 m*M* acetate buffer (pH 5.0) delivered at a flow-rate of 1 ml/min. The sample then entered the ECL flow-cell. Here, $Ru(bpy)_3^{2+}$ was converted to

Table 1
Important compounds in biological samples [26]

Compound	Concentration (μM)	
	Urine	Plasma
Oxalate	80–520	1.0–15.7
Histidine	500–1500	70–100
Proline	150–300	100–300
Tryptophan	0	35–75
3-Indoxylsulfuric acid	660–1170	2.8–25
3-Indole acetic acid	1.5–15	6.0–12.0

$Ru(bpy)_3^{3+}$ at the platinum electrodes and CL was initiated.

The interferants were studied in two groups, the amino acids and the indole-based compounds. Samples of these compounds were injected alone at different concentrations, and in mixtures with differing concentrations of interferant and a constant concentration of oxalate. Both the oxalate and interferants in the samples were at concentrations comparable to those found in biological samples.

2.4. Determination of oxalate in plasma and urine by HPLC

The reversed-phase ion-pair HPLC technique was carried out according to the procedure of Fry and Starkey [15]. The analytical column was conditioned by pumping 200 ml of the column conditioning solution to waste at 1 ml/min. This was followed by pumping 200 ml of mobile phase at 1 ml/min. The column conditioning gave reproducible retention times for several months without need for further reconditioning. All experiments were done at room temperature and a mobile phase flow-rate of 1 ml/min with the $Ru(bpy)_3^{2+}$ CL reagent at a flow-rate of 0.25 ml/min.

Fresh urine samples were filtered using a 0.45-μm syringe filter. Samples were spiked with a stock oxalate standard for standard addition determination, and then diluted four-fold with mobile phase before analysis. Injection volumes were 25 μl for all samples. For comparison, the same urine samples were also assayed for oxalate using Sigma Diagnostics enzymatic test kits for determination of oxalate. These test kits are only approved for determination of oxalate in urine and not in blood. Quantitation is done by measuring absorbance at 590 nm.

Blood samples containing anticoagulant were centrifuged for 15 min and the plasma drawn off. The protein was removed from the plasma samples before analysis by centrifuging in Centrifree (Amicon Corp., Danvers, MA, USA) ultrafiltration protein removal tubes. Plasma samples were spiked with oxalate for standard addition determination and 25 μl injected onto the chromatographic setup.

3. Results and discussion

3.1. Amino acid interferant effect

Study of the interaction between oxalate and the amino acids histidine and proline shows that the CL intensity increases as any of the single component concentrations in the mixture is increased. Signal increases are proportional to the concentration added of either the amino acids or oxalate, with respect to working curves of each component. This was studied at all concentrations throughout the clinical range for these compounds. This kind of behavior was expected and is seen with most reported compounds that give chemiluminescence with $Ru(bpy)_3^{2+}$.

3.2. Indole interferant effect

There are many indole-based compounds present in blood and urine, including hormones, neurotransmitters, and precursors and metabolites of these compounds. A study of several of the indole compounds present in biological samples, including 3-indoxyl sulfuric acid (ISA) and 3-indole acetic acid (IAA) shows a CL interaction very different from those of the amino acids studied. Indole itself was also used in this study since it is the parent compound.

When these compounds were injected alone with $Ru(bpy)_3^{2+}$, an increase in CL intensity was observed as the interferant concentration was increased, until a maximum was reached at concentrations between 75 and 200 μM, dependent upon the specific compound tested. This data is shown in Fig. 4. If the concentration of indole compound was increased further, the CL intensity decreased and approached zero. As the indole concentration was increased past the maximum intensity, peak splitting was observed, where the intensity of the center of the peak decreased, much like self-absorbance. This effect was more pronounced with higher concentrations where the center of the peak decreased to near

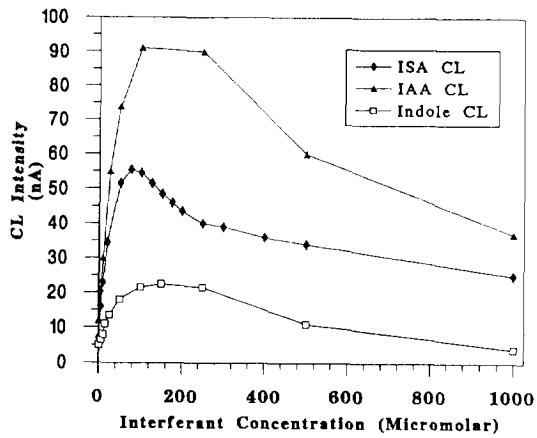

Fig. 4. Flow-injection analysis (FIA) chemiluminescence of interferants at pH 5.0 with 500 μM $Ru(bpy)_3^{2+}$ in samples.

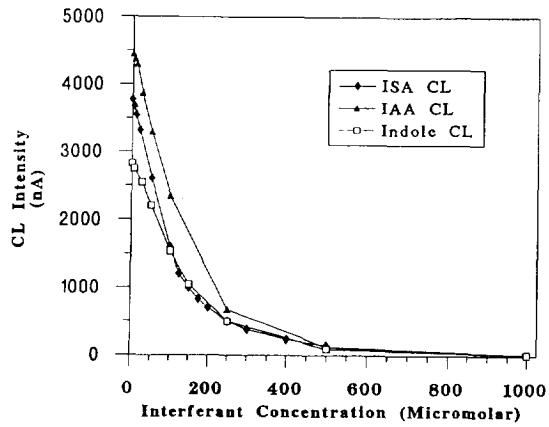

Fig. 5. Effect on CL from adding interferants to samples containing constant concentration of oxalate at 300 μM with 500 μM $Ru(bpy)_3^{2+}$ in samples. Studied by FIA.

zero intensity. This behavior was seen with all indole compounds studied.

Fluorescence and absorbance experiments were done with the indole compounds and $Ru(bpy)_3^{2+}$ to see if the effect was caused by a direct interaction with $Ru(bpy)_3^{2+}$. Solutions all containing 500 μM of $Ru(bpy)_3^{2+}$ and differing concentrations of ISA ranging from 0 to 1000 μM were used for fluorescence experiments. An excitation wavelength of 525 nm was used and the fluorescent emission from $Ru(bpy)_3^{2+}$ was monitored at 585 nm. There was no significant change in fluorescence intensity (or in the shape of the fluorescence emission spectrum) with any of the ISA concentrations used. ISA therefore does not interfere with the fluorescence emission of $Ru(bpy)_3^{2+}$. Absorbance measurements also show that the indole compounds do not significantly absorb at the $Ru(bpy)_3^{2+}$ CL/fluorescence emission wavelength; the only significant ISA absorbance is at about 300 nm.

The indole interferants were then used with oxalate to evaluate their effect on oxalate determination. The oxalate concentration was held constant while the concentration of the interferants varied. This was done for oxalate concentration ranges typically found in urine and plasma, with the interferant concentrations also covering the range found in these samples. This data is shown in Figs. 5 and 6. A decrease in intensity is observed as the interferant concentration is increased. The previously observed peak splitting for FIA of oxalate/indole mixtures is now understood to be caused by increased emission suppression at the higher indole concentration in the peak center. The CL emission decrease is more noticeable in the urine oxalate concentration range, but is also significant in the plasma oxalate concentration range. Even a small amount of indole interferant present can cause a large decrease in intensity. For example, a typical urine oxalate concentration in the

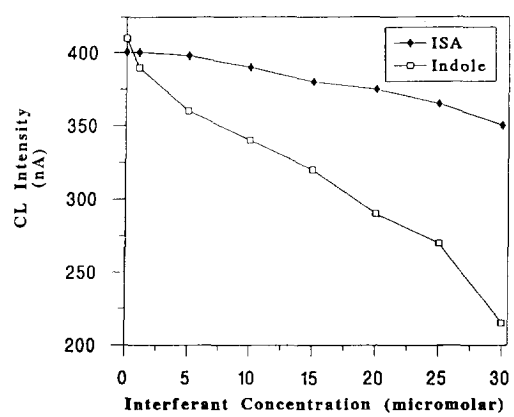

Fig. 6. Effect on CL from adding interferants to samples containing constant concentrations of oxalate at 10 μM with 500 μM $Ru(bpy)_3^{2+}$ in samples. Studied by FIA.

presence of 500 μM ISA would give a signal only about 5% of the signal obtained without the presence of interferant.

To see if this ECL signal suppression could be due to a specific interaction between oxalate and the indoles, oxalate was replaced with proline, which also gives intense CL emission with $Ru(bpy)_3^{2+}$. The results were the same for proline/indole mixtures as with oxalate/indole mixtures. With a constant concentration of proline, the CL emission intensity decreases as the indole concentration increases.

It should be noted that the behavior seen for indole compounds with $Ru(bpy)_3^{2+}$ ECL is unique in two respects: (1) the ECL emission intensity for an indole alone does not increase monotonically with the indole concentration, but passes through a maximum and decreases to essentially zero at higher concentrations; (2) the ECL emission intensity for a constant concentration of an analyte (such as oxalate or proline) decreases as the indole concentration increases even though that concentration of the indole alone would have resulted in ECL emission. This behavior has never been reported for any other compound tested with $Ru(bpy)_3^{2+}$.

Due to this unique interference caused by indoles, it was concluded that FIA determination with a simple sample cleanup was not sufficient, and chromatographic separation of oxalate from these interferants was necessary for accurate determination of oxalate.

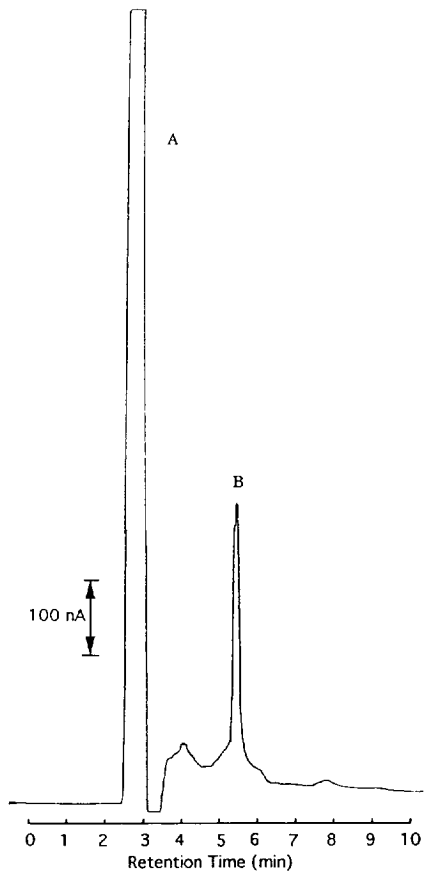

Fig. 7. Isocratic separation of oxalate in urine with $Ru(bpy)_3^{2+}$ CL detection on Zorbax ODS column (5 μM) with mobile phase containing 10% methanol in 100 mM phosphate buffer (pH 7.0). Mobile phase flow-rate = 1.0 ml/min; 25 μl urine injected. Retention times: (A) = amino acids ≈ 2:55 (min:s); (B) = oxalate ≈ 5:20.

3.3. HPLC determination of oxalate in urine

Using the conditions described in the experimental section, urine and plasma samples were assayed for oxalate using reversed-phase ion-pair HPLC. Standard addition was used to correct for matrix effects. Peak shapes and heights for oxalate injections varied for aqueous oxalate standards and actual biological samples, so standard addition was used instead of a calibration curve. Fig. 7 shows the chromatographic separation for a urine sample. The amino acids proline and histidine elute at retention times (t_r) of 2:45 (min:s) and 2:55 respectively as determined by injecting standards. In a mixture, they elute as a single peak which goes off scale due to their relatively high concentration in the samples. Oxalate is cleanly resolved, has a t_r of 5:20, and shows a sharp peak. The identity of other peaks in the chromatogram was not determined.

ISA injected alone gave no apparent peak, so some concern was given as to where this compound was eluting. This was investigated with synthetic samples containing oxalate, ISA and the amino acids. Changing the ISA concentration in the sample did not effect the oxalate peak at all, so it was determined that ISA did not

interfere with oxalate determination. By eliminating the analytical column, it was seen that ISA showed significant retention even on the very short guard column under these conditions.

Oxalate was quantitated by measuring the peak height above the average baseline signal over the peak width. For standard addition samples, oxalate peak heights were corrected for dilution. Oxalate concentrations determined for various samples gave reasonable values which fell in the normal clinical range. The standard addition plot for three samples is shown in Fig. 8.

3.4. HPLC determination of oxalate in plasma

Plasma samples were prepared as described in the Experimental section and injected for HPLC analysis. The separation of oxalate in a plasma sample is shown in Fig 9. Again, the amino acids elute at ≈2:50 and oxalate elutes at ≈5:20. Standard addition was used and oxalate peak heights were corrected for dilution. Detection limits were determined to be below 1 μM.

Oxalate values obtained using this technique were somewhat higher than the average clinical range of approximately 1.0–15.7 μM [26]. Oxalate determined in plasma samples ranged from 23 to 28 μM. It is well known that oxalate is produced by oxalogenisis in biological samples from conversion of ascorbic acid and its metabolites [3,27–29], glyoxylate [3,6,8,30], and other compounds present in biological samples. The blood samples used here were obtained from an outside source and were several days old when we received them. No steps had been taken to prevent conversion of these compounds to oxalate, which explains why the oxalate concentrations determined in our plasma samples were higher than normal.

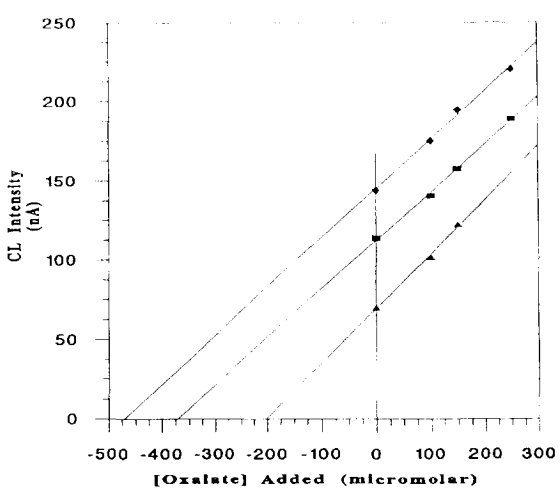

Fig. 8. Standard addition plots for determination of oxalate in three urine samples. Concentrations determined were 202.2, 371.0 and 470.6 μM. Samples were spiked with oxalate and peak heights corrected for dilution.

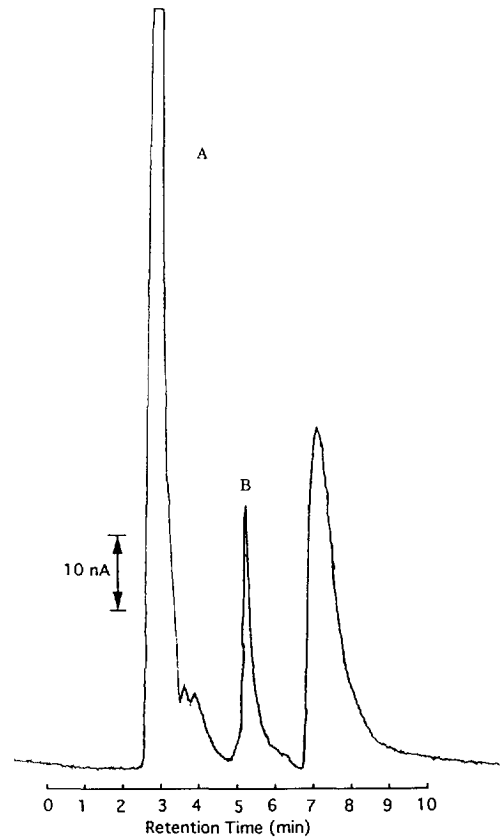

Fig. 9. Isocratic separation of oxalate in plasma with $Ru(bpy)_3^{2+}$ CL detection on Zorbax ODS column (5 μM) with mobile phase containing 10% methanol in 100 mM phosphate buffer (pH 7.0). Mobile phase flow-rate = 1.0 ml/min; 25 μl plasma injected. Retention times: (A) = amino acids ≈2:55 (min:s); (B) = oxalate ≈5:20.

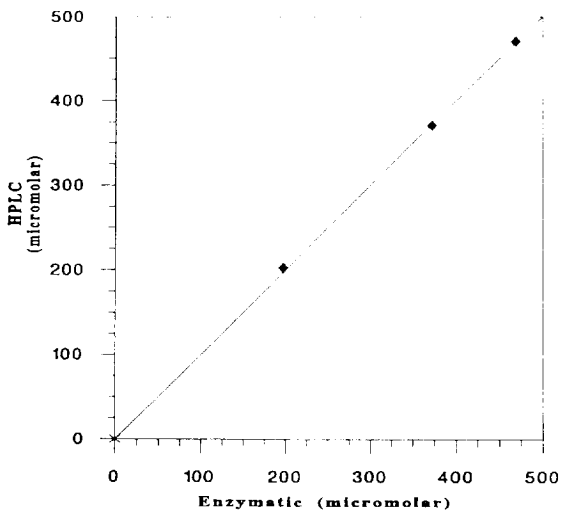

Fig. 10. Correlation of results from HPLC oxalate determination method for urine versus enzymatic urine oxalate kit (Sigma Chemical Co., procedure no. 591). Data shown in Table 2.

3.5. Comparison of oxalate in urine as measured with a commercial test kit

To compare our results for oxalate in urine with a widely accepted assay method, we used an oxalate enzymatic test kit. Urine samples were assayed by this method and by the $Ru(bpy)_3^{2+}$ HPLC method at the same time. The correlation results are shown in Fig. 10. The agreement between the two methods is very good. The data for the comparison is shown in Table 2. The average difference in the two assay values is 1%. The comparison could only be made with urine since the enzymatic test kits are not approved for analysis on blood.

Table 2
Comparison of urine oxalate concentrations determined by HPLC with $Ru(bpy)_3^{2+}$ ECL and by enzymatic test

HPLC with $Ru(bpy)_3^{2+}$ ECL (μM)	Enzymatic (μM)
202.2	197.3
371.0	372.6
470.6	468.5

4. Conclusions

This HPLC detection method for the determination of oxalate in urine and plasma samples shows many advantages over previously reported techniques. $Ru(bpy)_3^{2+}$ CL detection of oxalate covers a wide linear dynamic range covering the entire urine and plasma clinical concentration ranges. Detection limits are below 1 μM (1 nmol/ml) or 25 pmol injected, which makes this technique suitable for analysis in urine, and also for plasma samples which have much lower concentrations of oxalate. Detection limits for this method are substantially better than techniques using UV absorbance, and show better correlation with enzymatic tests on urine samples than techniques utilizing electrochemical detection. Sample preparation is extremely minimal for this technique and involves no derivatization. Sample analysis is fairly fast, requiring about 10 min per assay. The method could be modified to have the $Ru(bpy)_3^{2+}$ reagent immobilized on the electrode [18] or perhaps included in the mobile phase. These possibilities would eliminate the need for a second pump, a CL reagent stream and mixing tee, which may improve detection limits further. This HPLC system can also be used to assay for a wide range of compounds detectable with $Ru(bpy)_3^{2+}$, including other biological compounds as well as compounds in non-biological samples.

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