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Note

Profiling of oxalic acid and α-keto acids in blood and urine by liquid chromatography with electrochemical detection at a chemically modified electrode

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Elevated blood and urine levels of oxalic acid and α-keto acids have been associated with the occurrence of numerous human metabolic disorders. In particular, these compounds have been linked to the presence of several diseases of the newborn [1]; and, in addition, it has been suggested that high oxalate levels in urine exhibit a useful correlation for kidney stone formation [2,3]. Thus, the availability of convenient and reliable analytical methods for profiling these compounds in physiological samples would be of considerable diagnostic utility [4,5]. Unfortunately, oxalic acid and α-keto acids do not as a group represent ideal candidates for direct determination by conventional instrumental approaches. Usually, procedures employing gas and liquid chromatography (LC) [6–9] have been suggested; however, the volatility constraints of the former and the lack of a strongly absorbing UV–visible chromophore suitable for the optical detection methods most often employed in the latter severely restrict the direct applicability of these methods. As a result, preliminary derivatization of the acid analytes has routinely been required for either approach to be widely effective. Because the need for such steps can drastically effect the efficiency, convenience, and speed of the resulting analyses, the development of direct separation and detection techniques is desirable.

Electrochemical detection (ED) of oxalic acid and the α-keto acids utilizing their oxidation at glassy carbon electrodes has been reported to provide a potentially attractive LC detection scheme [10,11]. However, the potential required to effect the oxidations at conventional electrodes was of the order of +1.2 V vs. Ag/AgCl — which is sufficiently high to compromise both the sensitivity and the selectivity of the approach for real sample analysis. Recent work in our laboratory

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has demonstrated that electrocatalytic chemically modified electrodes (CMEs) can dramatically lower the potential required for detection of numerous difficult-to-oxidize analytes [12–14]. In particular, electrodes containing surface-bound cobalt phthalocyanine (CoPC) can decrease the potential required for oxalic and α-keto acid oxidation by roughly 0.5 V [14]. As a result, the sensitive detection and quantitation of the compounds in complex matrices may be possible without the need for initial derivatization or any elaborate sample preparation steps. In this work, the compatibility of the CoPC CMEs for extended use in the analysis of physiological samples and their capabilities for direct profiling of the oxalic acid and α-keto acid content of urine and blood are demonstrated.

EXPERIMENTAL

The CMEs employed were constructed as described previously [14] by thoroughly mixing an appropriate quantity of CoPC (Eastman Kodak) with 5 g of graphite powder and 3 ml of Nujol oil to form a modified carbon paste that could be packed into a conventional thin-layer electrochemical flow-cell with an Ag/AgCl reference electrode. Optimum performance was obtained with a 2.0% (or 0.10 g) CoPC loading. Chromatography was performed with a 25 cm$\times$4.6 mm I.D., 5-μm octadecylsilane column (Alltech) with a 0.10 *M* pH 2.0 phosphate buffer as mobile phase. The instrumental set-up was the same as that used in earlier work [14]. The injection volume was 5 μl, and the flow-rate was 1.0 ml/min.

Urine samples, obtained from healthy volunteers or from diagnosed kidney stone patients, were treated only by filtration with a 10–15 μm glass filter and dilution with mobile phase. Blood plasma samples, obtained from the Louisville Red Cross, were treated by first precipitating protein with trichloroacetic acid. To accomplish this, 2.5 ml of plasma and 0.5 ml of trichloroacetic acid were placed in 75$\times$12 mm polyethylene sample tubes and centrifuged thoroughly. The supernatant was suitable for injection onto the chromatograph through a Cameo Nylon filter (Micron Separations, Honeoye Falls, NY, U.S.A.) for particulate removal.

RESULTS AND DISCUSSION

At conventional carbon paste or glassy carbon electrodes, oxalic acid undergoes oxidation at a potential of +1.2 V vs. Ag/AgCl, while the α-keto acids require still higher values. However, when CoPC is incorporated onto the electrode surface, the oxidation waves are shifted to considerably lower potentials, the oxalic acid wave appearing at +0.75 V and those for the α-keto acids at +0.90 V [14]. As a consequence, LC–ED of these analytes can be performed at CoPC-containing CMEs at potentials nearly 0.5 V lower than at conventional carbon electrodes. A decrease of this magnitude in LC–ED operating potential should have direct practical consequences for the determination of these compounds in complex sample matrices. In particular, the lower potential permitted by CME usage should decrease the number and extent of possible interferences, thereby simplifying the sample treatment and chromatographic procedures required. In this case, the

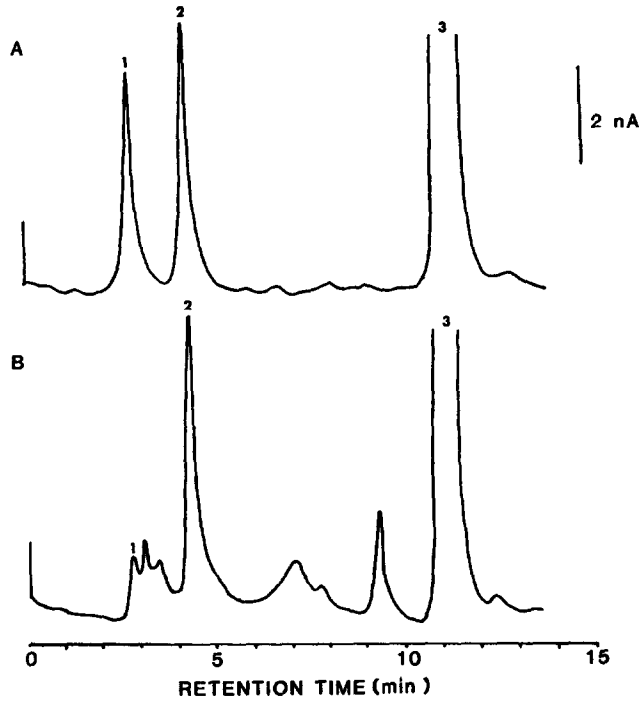

Fig. 1. Chromatograms of (A) 100 μl of urine diluted to 50 ml at 2.0% CoPC electrode at +0.75 V vs. Ag/AgCl and (B) same sample as in A but obtained at a plain carbon paste electrode at +1.1 V. Peaks 1, 2, and 3 correspond to oxalic acid, ascorbic acid, and uric acid, respectively.

selectivity enhancement was sufficient to permit direct profiling of oxalic acid and α-keto acids in both human urine and blood serum.

A typical chromatogram obtained at a CoPC CME held at +0.75 V is shown in Fig. 1A for a urine sample collected from a healthy volunteer. The chromatogram exhibited three principal peaks whose retention times matched those of oxalic acid (3.0 min), ascorbic acid (5.0 min), and uric acid (12.3 min). This is not surprising since appreciable concentrations of all three compounds are known to be present in such samples [15]. Note that the latter two compounds are oxidized at very low potentials and thus can be detected without difficulty even at unmodified electrodes. However, no response at all was observed for oxalic acid at this potential when an unmodified carbon paste or glassy carbon electrode was employed. Rather, with these conventional electrodes, much higher potentials were required. As shown in Fig. 1B, only a comparatively small oxalic acid peak was obtained at plain paste even at +1.1 V vs. Ag/AgCl. Furthermore, when these high potentials were employed, several additional peaks were in evidence, some overlapping seriously with that of the analyte and making its quantitation under these circumstances tenuous at best.

At the CoPC CME, the detection limit (signal-to-noise ratio 2) for oxalic acid at +0.75 V was 0.30 pmol injected. The addition of several α-keto acids, not present at appreciable levels in normal urine samples but useful diagnostically for several disorders of the newborn, produced analytically useful peaks well

TABLE I

URINARY OXALATE BY LC-ED AT CoPC CME

Values represent the mean of three measurements; reproducibility is shown as the standard deviation of these determinations.

Normal samples		Kidney stone patient samples	
Volunteer	Oxalate (mg per 24 h)	Patient	Oxalate (mg per 24 h)
A	18.9±0.2	A	28.3±0.9
B	22.3±0.4	B	29.5±0.4
C	25.6±0.8	C	45.2±0.6
D	15.8±0.5		

resolved from all background signals. Detection limits for the α-keto acids ranged from 0.10 to 1.0 nmol [14].

Previous reports by several groups have claimed that mild hyperoxaluria occurs in 30–40% of all patients with idiopathic renal stones and that elevated oxalate in the urine correlates more positively with the disease than does the more commonly used calcium level [2,3]. Table I lists the results of triplicate oxalic acid determinations performed on urine samples from both healthy individuals and diagnosed kidney stone patients. In all cases, the values resulting from these standard addition determinations fell within the ranges reported for normal and abnormal subjects. Even though sample treatment procedures consisted of only particulate filtration, no co-eluting interferents were detected with any of the urine samples examined.

Oxalic and pyruvic acids are also known to be normal constituents of blood plasma, with elevated levels associated with disorders such as pyruvic acidemia [1]. Therefore, chromatograms, shown in Fig. 2 and summarized for several samples in Table II, were also obtained for plasma samples from healthy subjects. Again, in all four cases examined, no co-eluting interferents were observed and the oxalic and pyruvic acid values obtained fell close to the expected normal range (1.4–2.8 mg/l for oxalic acid [15] and 4–20 mg/l for pyruvic acid [16]). The sole sample treatment employed consisted of protein precipitation, centrifugation, and filtration; this permitted greater than 99% recovery of both analytes with total analyses times of the order of 10–15 min. Because of the CoPC CME's much greater response toward oxalic acid at the +0.75 V applied potential, a greater dilution of the plasma sample was used for determination of this component.

The stability of the CME response was tested by placing the CoPC-containing electrode in the flowing mobile phase stream and following its response for repeated injections of a plasma sample over an extended length of time. As long as the electrode was maintained at the +0.75 V vs. Ag/AgCl or lower, peak heights observed for the analytes were practically unchanged — with the CME retaining more than 95% of its initial activity after 3 h of continuous use. Even better stability was observed when urine samples were employed in place of plasma. Operation at a higher potential did cause a much more rapid decrease in electrode response and thus should be avoided in practice. In any case, one of the virtues

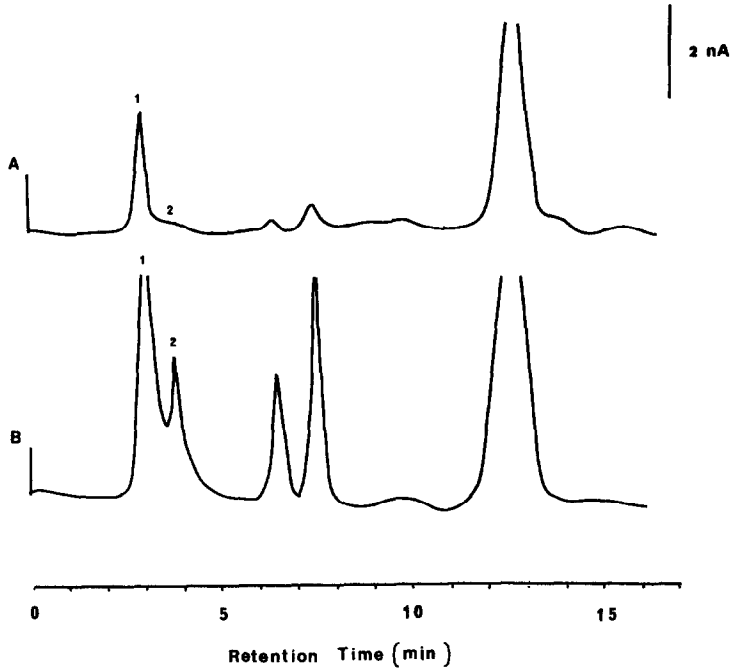

Fig. 2. Chromatograms of (A) 5.0% blood plasma at 2.0% CoPC electrode and (B) 70.0% blood plasma at the same electrode. Peaks 1 and 2 correspond to oxalic acid and pyruvic acid, respectively. $E = +0.75$ V vs. Ag/AgCl.

of the modified carbon paste surface is that, if necessary, it can be rapidly and reproducibly renewed by simply removing the exposed layer of paste and smoothing on a fresh portion.

In all respects, the LC–ED/CME approach for quantitation of oxalic acid and the α-keto acids seems to be considerably more attractive for blood and urine profiling than previously suggested methods. Absolute detection limits are quite low. Because of the lower detection potential made possible by incorporation of the CoPC modifier, system selectivity is sufficiently high that no derivatization and only minimal sample treatment are required. Finally, the CMEs are quite

TABLE II

OXALIC AND PYRUVIC ACID IN PLASMA BY LC-ED AT CoPC CME

Values represent the mean of three measurements; reproducibility is given as the standard deviation of these determinations.

Sample	Concentration (mg/l)	
	Oxalic acid	Pyruvic acid
A	1.2±0.2	9.4±0.5
B	1.6±0.1	14.1±0.8
C	2.4±0.3	21.1±0.2
D	2.0±0.5	19.0±0.8

durable, maintaining a virtually constant level of response over hours of continuous usage in the chromatographic stream and exposure to numerous urine or plasma injections.

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