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Amperometric determination of oxalate in plasma and urine by liquid chromatography with immobilized oxalate oxidase

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Abstract

A detection system for plasma and urinary oxalate involving a postcolumn enzymatic reaction and electrochemical detection is described. Oxalate oxidase was immobilized on AF-Tresyl Toyopearl 650 gel. The immobilized oxalate oxidase was packed into a stainless-steel column (10 × 4 mm I.D.) and used on-line as an immobilized enzyme reactor (IMER). The hydrogen peroxide produced by enzymatic reaction was detected amperometrically at a platinum electrode maintained at +0.5 V *vs.* Ag/AgCl. The HPLC separation of oxalate was carried out using a Capcell Pak C_8 column and an isocratic mobile phase containing 80 m*M* KH_2PO_4 and 5 m*M* tetra-*n*-butylammonium phosphate as an ion-pair reagent. The IMER was active and stable in the mobile phase employed. The plot of peak height against concentration of oxalate was linear in the range 0.1–1.6 μmol/ml with a correlation coefficient of 0.9989. The detection limit was 10 nmol/ml at a signal-to-noise ratio of 3. The within-day and between-day coefficients of variation were 5.3 and 7.9%, respectively.

1. Introduction

The measurement of oxalate in plasma and urine is clinically important for the diagnosis of various disorders. In particular, the determination of urinary oxalate is of great significance in the investigation of renal stone formation [1,2]. Many methods for determining oxalate in biological fluids have been reported, including spectrophotometry [3], fluorimetry [4,5], gas chromatography [6] and ion chromatography with conductimetric detection [7]. High-performance liquid chromatography (HPLC) for the determination of oxalate has been described, in which oxalate was measured by UV spectrophotometry [8,9], fluorimetry [10] or amperometric detection with a copper electrode [11].

Enzymatic methods, which are based on the use of a soluble enzyme, either oxalate decarboxylase or oxalate oxidase, have also been reported for the determination of oxalate. The enzymatic products were measured by spectrophotometry [12–14] or pH measurement [15]. Another enzymatic approach for determining oxalate, in which oxalate oxidase was immobilized and used in a flow system, has recently been described. The oxalate was oxidized to hydrogen peroxide and carbon dioxide. The heat signal generated by the enzymatic reaction was directly measured in a calorimetric device, the enzyme thermistor [16], or the hydrogen peroxide formed was monitored by a spectrophotometric [17,18] or amperometric detector [19]. An en-

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zyme electrode, oxalate oxidase immobilized on an oxygen electrode, has also been employed for the determination of urinary oxalate [20]. The application of immobilized enzyme reactors (IMERs) as the reaction detection system in HPLC provides high sensitivity and analytical selectivity [21]. We have already proposed the HPLC system with IMER detection for determining acetyl-coenzyme A (CoA) [22], and reported its usefulness for the selective determination of acetyl-CoA in a mixture of short-chain acyl-CoA esters. This paper describes a specific detection system for plasma and urinary oxalate involving HPLC separation followed by an IMER postcolumn and amperometric detector for hydrogen peroxide.

2. Experimental

2.1. Materials and solutions

All chemicals used were of analytical-reagent grade. Oxalate oxidase (oxalate:oxygen oxidoreductase, EC 1.2.3.4, 0.34 U/mg) from barley seedlings was purchased from Boehringer (Mannheim, Germany). The ion-pair reagent, tetra-*n*-butylammonium phosphate (TBAP), was obtained from Nacalai Tesque (Kyoto, Japan), AF-Tresyl Toyopearl 650 gel ($-CH_2OSO_2CH_2-CF_3$ residue; 100 μmol/g dry gel) from Tosoh (Tokyo, Japan) and Bond Elut C_{18} cartridges from Varian (Harbor City, CA, USA). Solutions were prepared with doubly demineralized, distilled water. Stock standard solutions of dicarboxylic acids were prepared at a concentration of 10 m*M* of each acid in 0.1 *M* HCl and working standard solutions were then prepared by dilution with 0.05 *M* HCl to the required concentrations. The working standard solution of oxalate was prepared daily. The mobile phase contained potassium dihydrogenphosphate (KH_2PO_4) and TBAP as an ion-pair reagent and the pH was adjusted with 5% orthophosphoric acid (H_3PO_4). The mobile phase was filtered through 0.4-μm pore size filters (Fuji Photo Film, Tokyo, Japan) and degassed.

2.2. Preparation of enzyme reactor

Oxalate oxidase (7 mg) dissolved in 1.2 ml of 0.1 *M* phosphate buffer (pH 8.0) containing 0.5 *M* sodium chloride was immobilized on 150 mg of AF-Tresyl Toyopearl 650 gel by shaking occasionally for 12 h at room temperature. The subsequent procedure was the same as described previously [23]. The resulting immobilized enzyme was tightly packed into a stainless-steel column (10 ×4 mm I.D.) and used as the IMER. A blank column, enzymatically inactive, was prepared in a similar manner as reported earlier [22].

2.3. Sample preparation and storage

Plasma and urine samples were collected from healthy volunteers. Plasma from heparinized blood was deproteinized by the published procedure [15], except that 0.03 *M* HCl was used in place of 0.05 *M* HCl and merthiolate was not added. The supernatant, adjusted to pH 3.8, was pretreated by using a Bond Elut C_{18} cartridge.The clean-up treatment was useful for increasing the lifetime of the analytical column. Urine samples were cleaned up by the method of Larsson *et al.* [8], except that the urine was acidified with adding an equal volume of 0.09 *M* HCl. Acidified urine was pretreated by passing it through a Bond Elut C_{18} cartridge. The sample clean-up was completed in 1–2 min. The deproteinized plasma sample or the cleaned-up urine sample was stored at −20°C.

2.4. Apparatus and procedure

A schematic diagram of the flow system for the determination of oxalate is shown in Fig. 1. The chromatographic system consisted of a Shimadzu (Kyoto, Japan) LC-6A pump equipped with a Rheodyne (Cotati, CA, USA) Model 7125 syringe-loading sample injector with a 100-μl sample loop. Reversed-phase ion-pair chromatographic analysis was performed on a Capcell Pak C_8 column (150 × 6 mm I.D., SG type, 5-μm particle) (Shiseido, Tokyo, Japan). The separation column and the IMER were

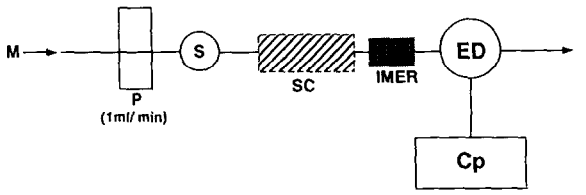

Fig. 1. Schematic diagram of the combined IMER–reversed-phase HPLC system. M = Mobile phase; P = pump; S = sample injector; SC = separation column; IMER = immobilized enzyme reactor; ED = electrochemical detector; Cp = computing integrator.

thermostated at 30°C in a Shimadzu CTO-6A column oven. For examination of the elution behaviour of dicarboxylic acids in reversed-phase ion-pair liquid chromatography, the IMER was removed from the flow system and the column effluent obtained with isocratic elution was monitored at 215 nm with a Shimadzu SPD-6AV spectrophotometer equipped with an 8-μl flow cell. When the hydrogen peroxide produced by the enzymatic reaction was measured, the IMER was included in the flow system, and the amperometric response was detected using an ECD 100 detector (Eicom, Kyoto, Japan) with a platinum working electrode. The working electrode potential was kept at +0.5 V *vs.* Ag/AgCl. Chromatographic data were recorded on a C-R4A integrator (Shimadzu). The mobile phase was pumped at a flow-rate of 1.0 ml/min and sample solutions of 50 μl were injected through a Rheodyne injector. After 1 week of use, the IMER was washed with water and the analytical column was washed with water and then 70% methanol at a flow-rate of 1.0 ml/min for at least 30 min.

3. Results and discussion

3.1. Chromatographic conditions

Organic modifiers in the mobile phase adversely affected the activity and stability of the IMER, as discussed previously [22]. A convenient HPLC technique, based on reversed-phase ion-pair chromatography, has been reported previously [8]. In that work, the separation was carried out with a mobile phase consisting of 36.7 m*M* KH_2PO_4 and 5 m*M* TBAP buffered to pH 2.00 with H_3PO_4. The pH of the mobile phase was low compared with the optimum pH for the immobilized oxalate oxidase [16,19]. Therefore, an investigation of the elution behaviour of oxalate on a Capcell Pak C_8 column was achieved prior to inclusion of the IMER on-line in the flow-system.

The capacity factors (k') of oxalate, together with those of other dicarboxylic acids, *i.e.*, malic, malonic, succinic, glutaric, adipic, fumaric and maleic acid, and uric acid were determined with mobile phases with different concentrations of KH_2PO_4 and TBAP and pH values. The k' value was determined from the retention time relative to the dead time, *i.e.*, t_0, of an unretained compound.

We examined two compounds, sodium nitrite and oxalate, as candidates for a non-retained standard. The mobile phase was 80 m*M* KH_2PO_4 buffered to various pH values with 5% H_3PO_4. The elution times (min) were 4.18 (pH 2.0), 3.57 (pH 3.5), 3.14 (pH 5.0), 3.10 (pH 6.0) and 3.09 (pH 7.0) for sodium nitrite and 3.15 (pH 2.0), 3.06 (pH 3.5), 2.92 (pH 5.0), 2.90 (pH 6.0) and 2.89 (pH 7.0) for oxalate. We observed that highly polar oxalate was not retained to the column in the absence of TBAP as a counter ion, and the apparent t_0 for this column was 2.89 min. The retention of dicarboxylic acids and uric acid was enhanced with the increasing amounts of TBAP (Fig. 2). The retentions were regulated by changing either the pH or the molarity of KH_2PO_4 in the mobile phase.

The influences of pH and the ionic strength on the k' values are shown in Figs. 3 and 4. The elution behaviour of the acids depends on their dissociation constants (pK_a).

As a result of careful examination, the resolution of a mixture of eight dicarboxylic acids was achieved by using a mobile phase consisting of 90 m*M* KH_2PO_4 and 5 m*M* TBAP at pH 4.0, and all acids were eluted within 30 min. The k' value for oxalate was 1.115. A typical chromatogram of the mixture is shown in Fig. 5.

For determining oxalate in plasma and urine, the use of 80 m*M* KH_2PO_4 was preferred to 90

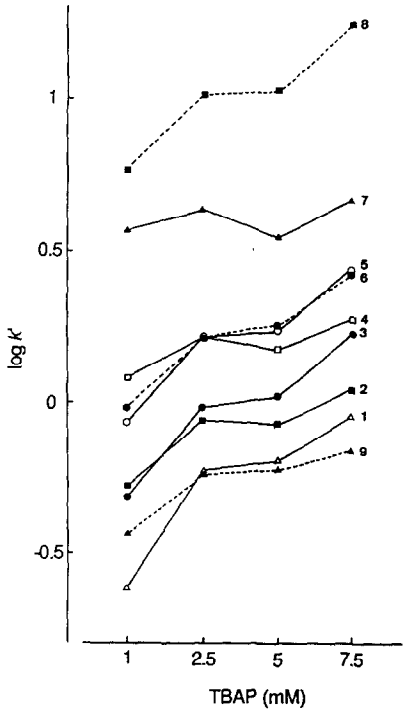

Fig. 2. Influence of the concentration of the ion-pair reagent (TBAP) in the mobile phase on the capacity factors (k') of dicarboxylic acids and uric acid. The mobile phase consisted of 75 mM KH_2PO_4 and the given concentration of TBAP, and buffered to pH 3.5 with 5% H_3PO_4. 1 = Malic acid; 2 = succinic acid; 3 = oxalic acid; 4 = glutaric acid; 5 = malonic acid; 6 = fumaric acid; 7 = adipic acid; 8 = maleic acid; 9 = uric acid.

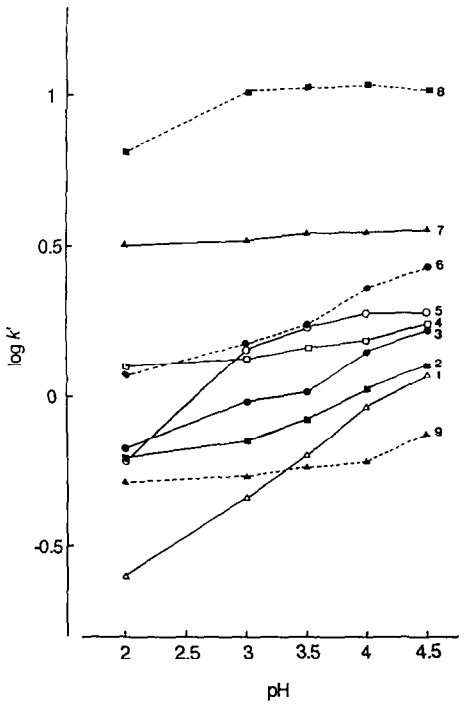

Fig. 3. Influence of pH of the mobile phase on the k' values of dicarboxylic acids and uric acid. The mobile phase consisted of 75 mM KH_2PO_4 and 5 mM TBAP, buffered to a given pH with 5% H_3PO_4. Line identifications as in Fig. 2.

mM KH_2PO_4 to avoid the chromatographic interference of electrochemically active substances such as ascorbic acid and uric acid. The maximum activity for the immobilized oxalate oxidase was found between pH 3.5 and 3.8 [16,19]. Consequently, a mobile phase consisting of 80 mM KH_2PO_4 and 5 mM TBAP buffered to pH 4.0 with H_3PO_4 was the best selection for this detection system.

3.2. Calibration

The enzymatically formed hydrogen peroxide was detected electrochemically under the flow system. The amperometric response was quantitatively obtained by injecting samples containing various amounts of oxalate. The plot of peak height *versus* the concentration of oxalate was linear in the range 0.1–1.6 μmol/ml, which is adequate for clinical analyses. The regression equation and the correlation coefficient (r) for peak height (y, mV) *versus* concentration (x, μmol/ml) were $y = 11.459x - 0.031$, $r = 0.9989$ ($n = 18$). The detection limit at a signal-to-noise ratio of 3 was 10 nmol/ml.

3.3. Application to plasma and urine samples

Figs. 6 and 7 show chromatograms of plasma and urine samples, respectively, obtained using the HPLC system with and without IMER detection. Two typical chromatograms for different plasma samples, with low (Fig. 6a) and high (Fig. 6b) concentrations of oxalate were obtained. The oxalate peak at a retention time of 9.4 min was detected well separated from other electrochemically active substances, as shown, the peaks eluting earlier being mainly ascorbic

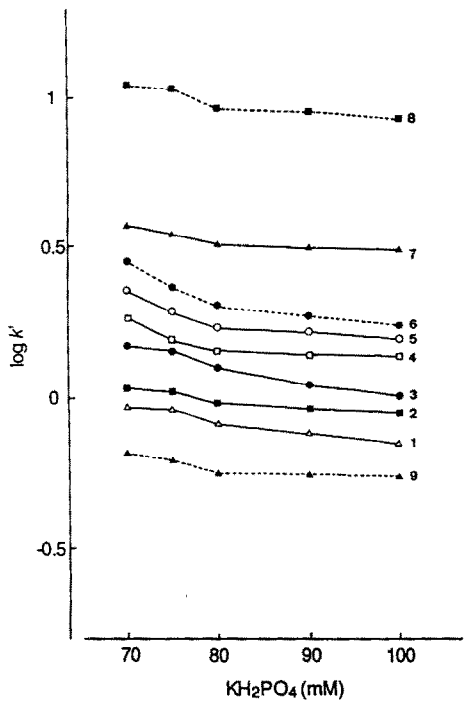

Fig. 4. Influence of the molar concentration of KH_2PO_4 in the mobile phase on the k' values of dicarboxylic acids and uric acid. The mobile phase consisted of the given concentration of KH_2PO_4 and 5 mM TBAP, buffered to pH 4.0 with 5% H_3PO_4. Line identifications as in Fig. 2.

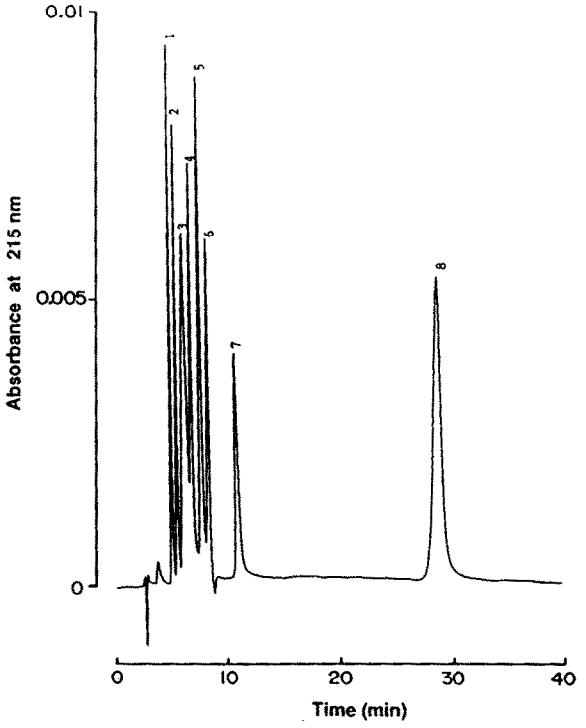

Fig. 5. Chromatographic separation of a mixture of eight dicarboxylic acids by reversed-phase ion-pair HPLC. The mobile phase consisted of 90 mM KH_2PO_4 and 5 mM TBAP at pH 4.0; flow-rate, 1.0 ml/min; temperature, 30°C. Peaks: 1 = malic acid ($2 \cdot 10^{-4}$ M); 2 = succinic acid ($4 \cdot 10^{-4}$ M); 3 = oxalic acid ($1 \cdot 10^{-4}$ M); 4 = glutaric acid ($4 \cdot 10^{-4}$ M); 5 = malonic acid ($4 \cdot 10^{-4}$ M); 6 = fumaric acid ($2 \cdot 10^{-6}$ M); 7 = adipic acid ($4 \cdot 10^{-4}$ M); 8 = maleic acid ($8 \cdot 10^{-6}$ M).

and uric acid. Fig. 6c is a chromatogram of a spiked plasma sample to which oxalate at a final concentration of 0.30 μmol/ml was added with a high concentration of oxalate. The oxalate peak increased with increased addition of oxalate.

The chromatograms shown in Fig. 7a and b are also representative of urine samples with different amounts of oxalate. The urine samples contained low (Fig. 7a, 0.1 μmol/ml) and high (Fig. 7b, 0.3 μmol/ml) concentrations of oxalate. The peaks eluting earlier were much larger than those for plasma samples. Fig. 7c shows a chromatogram of a spiked urine sample to which oxalate at a final concentration of 1.0 μmol/ml was added with a high concentration of oxalate. The oxalate peak increased in proportion to the amount of oxalate added.

When sample solutions identical with those used in Figs. 6c and 7c were injected into the system and the blank column was used in place

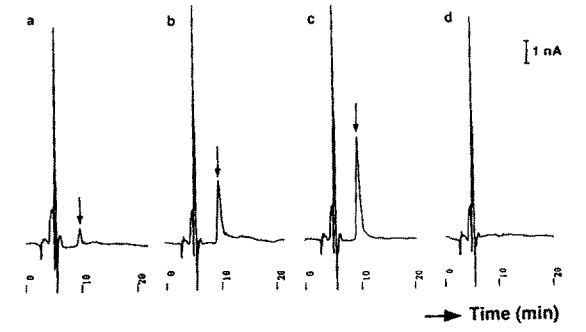

Fig. 6. Chromatograms of plasma samples obtained using the HPLC system (a–c) with and (d) without IMER detection. The samples contained different amounts of oxalate; (a) low concentration; (b) high concentration; (c) and (d) plasma sample with high concentration of oxalate spiked to a final concentration of 0.3 μmol/ml. The arrows indicate the oxalate peak.

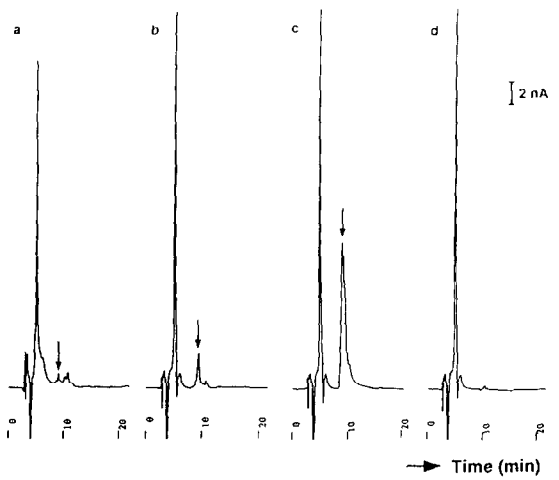

Fig. 7. Chromatograms of urine samples obtained using the HPLC system (a–c) with and (d) without IMER detection. The samples contained different amounts of oxalate: (a) low concentration; (b) high concentration; (c) and (d) urine sample with high concentration of oxalate spiked to a final concentration of 1.0 μmol/ml. The arrows indicate the oxalate peak.

of the IMER, no oxalate peaks were detected (Figs. 6d and 7d).

Table 1 shows the analytical recovery of oxalate from plasma and urine. Different oxalate concentrations ranging from 0.2 to 1.2 μmol/ml were added to a known amount of plasma or urine sample. The mean recovery was 86.7% for plasma and 95.9% for urine samples. The percentage recovery from plasma was slightly lower than that in previous work [15], but that from urine was equivalent to others [5,7–11,13,16,18,20]. The within-day precision was determined seven times each on the same day with a given urine sample (0.53 μmol/ml). For determining the between-day precision, a urine sample identical with that employed for within-day assay was measured once on each of five days. The relative standard deviation (R.S.D.) of the within-day and the between-day precision was 5.3% and 7.9%, respectively. Sample preparation of urine was easy, and the analytical recovery and the precision were also satisfactory.

Table 1
Recovery of oxalate added to plasma and urine

Sample	Oxalate added (μmol/ml)	Oxalate found[a] (μmol/ml)	Recovery (%)
Plasma	0.2	0.195	97.5
		0.190	95.0
	0.4	0.362	90.5
		0.374	93.5
	0.6	0.516	86.0
		0.512	85.3
	1.2	0.919	76.6
		0.826	68.8
Mean			86.7
Urine	0.2	0.197	98.5
		0.224	112.0
	0.6	0.559	93.2
		0.579	96.5
	1.2	1.014	84.5
		1.091	90.9
Mean			95.9

[a] The amount determined was corrected for oxalate already present.

The immobilized oxalate oxidase was stable. The IMER has been used continuously at 30°C for 4 months for more than 400 samples without any significant decrease in activity. Hence the proposed method may have the simplicity and specificity needed for determining oxalate in biological fluids, especially urine.

4. Acknowledgement

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5. References

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