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Use of Citric Acid as Mobile Phase and Sample Medium for the Determination of Ascorbic Acid by Liquid Chromatography/Electrochemistry

The use of citric acid (20 mM, pH ca. 2.5) in both the mobile phase and sample medium was examined for determination of ascorbic acid (AA) in foods and biological fluids by liquid chromatography/electrochemistry. Background current due to mobile phase and the relationship between applied potential and sensitivity to AA were examined. The applied potential was set at +400 mV versus Ag/AgCl.

Citric acid proved useful both in mobile phase and sample medium for determination of AA in foods and biological fluids. A large volume of aqueous solution (pH 2.5) could be prepared simply and rapidly without need for further pH adjustment. The proposed method was simple, rapid (retention time: ca. 6.4 min), sensitive (detection limit: ca. 0.1 ng per 20 µL injection at a signal-to-noise ratio of 3), selective and reproducible (relative standard deviation 3% (n = 5)). The response was linear in the range of 0.1 to 16 ng per injection. Recovery of AA was over 90% by the standard addition method.

Mobile phase containing inorganic salts such as potassium phosphate and ethylenediaminetetraacetic acid (EDTA), with pH adjustment are often used for determination of ascorbic acid (AA) in foods and biological fluids by liquid chromatography/electrochemistry (LC/EC).

Metaphosphoric acid (MPA), which has long proved its worth as a sample medium, was selected for determination of AA due to its ability to stabilize samples before analysis. Many reports on LC determination of AA have appeared (1-16), and Oliveira and Watson (17) have reviewed the chromatographic techniques for determination of putative dietary anticancer compounds (including AA) in biological fluids. Evaluation of sampling and extraction procedures for determination of AA in pear fruit tissue has also been reported (15). It is essential to avoid high temperature and light exposure during sample preparation. Moreover, AA is easily oxidized in the presence of certain reagents such as halogens, hydrogen peroxide and heavy metal ions, especially Cu^{2+}, Fe^{3+} and alkaline pH (8).

In routine work, large volumes of MPA aqueous solution and mobile phase must be prepared. These both present the following problems:

1. MPA dissolves very slowly in deionized water.
2. MPA is hygroscopic and exists in more than one physical form, so accurate weighing is not easy.
3. pH adjustment (pH 2-3) of mobile phase is tedious.

Previous papers (12-14) have indicated that it was possible to replace MPA in the mobile phase with, for example, potassium phosphate with EDTA, monosodium L-glutamate (MSG) and disodium guanosine-5'-monophosphate (GMP) aqueous solutions after pH adjustment. These compounds chelate some metal ions, enhancing the pre-analysis sample stabilization of AA in foods. This avoids problems with weighing and dissolution of MPA. However, the pH must still be adjusted, so we looked for a chelating agent that can be used in both mobile phase and sample medium, and that can be prepared simply and rapidly without need for further pH adjustment.

This paper deals with the stability of AA in human urine and standard diluted with tap water containing other compounds which might also be present in foods or human urine, in the search for a suitable mobile phase and sample medium. It also concerns the possible use of citric acid (20 mM, pH ca. 2.5) in both mobile phase and sample medium for determination of AA in foods and biological fluids by LC/EC. In addition, we have compared the background current obtained from the proposed mobile phase with those generated by mobile phases recommended in the literature, and the relationship between applied potential and response to AA and uric acid (UA). A healthy human volunteer took both breakfast and lunch (potato salad, orange juice, Japanese tea, etc.), after which urine was periodically analyzed over a 12 h period to examine the relationship between urine AA level and time after food consumption. In addition, the proposed method was applied to determination of AA in foods and other biological fluids.

Experimental

Reagents and Materials

AA and citric acid used in this study were purchased from Wako (Osaka, Japan). Other reagents were all of analytical grade. Light-resistant brown

volumetric flasks and glassware were used (8-12). The membrane filters (HLC-DISK 25, 0.45 µm, polyvinyl-difluoride) were purchased from Kanto Kagaku, Tokyo, Japan.

Standard AA Preparation

Standard AA (100-800 ng/mL) was made up in mobile phase in a brown volumetric flask just prior to use.

Sample Preparation

Samples were first diluted to an estimated AA concentration of 100-800 ng/mL in a brown volumetric flask with mobile phase, and then passed through a membrane filter (0.45 µm). The filtrate (over 1 mL) was used for determination of AA.

LC Conditions

A model 655 A-11 liquid chromatograph (Hitachi, Tokyo) equipped with a Model ED 623 electrochemical detector (working electrode: glassy carbon, GL Sciences, Tokyo) was used. The applied potential was set at +400 mV, versus an Ag/AgCl reference electrode. The samples were applied using a Rheodyne Model 7125 injector with 20 µL loop. LC was carried out in a 15 X 0.46 cm I.D. reversed-phase column (Inertsil ODS-3, 5 µm, GL Sciences) with guard column of Inertsil ODS-3, 5 µm (1 X 0.46 cm I.D.), using 20 mM citric acid (pH 2.5) as the mobile phase at room temperature. The flow-rate was 0.6 mL/min.

Results and Discussion

Stability of AA in Human Urine

It is important to examine the stability of AA in samples stored at room temperature. The sample medium should keep AA stable as long as possible.

In an earlier paper (11), it was shown that AA in instant powdered soup prepared with hot tap water according to the label was very stable, even though this was in a neutral pH solution. It was suspected that other compounds in the sample might stabilize AA, so the present report also describes the stability of AA in biological fluids to examine this possibility.

AA in urine sample (pH ca. 6.1) stored in a brown flask was stable for 5h at room temperature. In comparison, for standards of AA (pH 6) at 25 ºC and 40 ºC, the concentrations were reduced to 95.6% and 70.9% (13), respectively, after one hour. This strongly suggests the presence of stabilizing compounds in urine. These data further suggest that we have enough time for the sample preparation of AA in human urine. However, AA was not stable in both tears and sweat samples, although stored in a brown flask. In particular, AA levels in sweat decreased rapidly.

Again, it is interesting that AA is present in human blood (pH ca. 7.4, temperature ca. 36-37 ºC) and urine (pH ca. 5-8, temperature ca. 36-37 ºC), considering that standard AA under these conditions is not normally stable (***T1***). In particular, standard AA diluted at pH 7.4 is quite unstable at 40 ºC. This suggests that co-existing compounds in the human body might be important in stabilizing AA.

These results led us to investigate the effect of some (co-existing) compounds on the stability of standard AA solutions.

Effect of Some Co-existing Compounds on the Stability of Standard AA

Attention was given to the effect of adding compounds (20 µM) suspected of being present in samples, on the stability of standard AA (2.5 µM) solutions in tap water stored in a brown flask at 25 ºC. The goal was to find the most effective mobile phase and sample medium for pre-run sample stability (***T1***).

In the present study, tap water, which contains anions, cations and residual chlorine, was used because it is often used for washing glassware and used for cooking and cleaning up. Data in ***T1*** suggest the existence of compounds that give a stabilizing effect. A summary is shown below:

1. Tap water: AA diluted in tap water was quite unstable, probably due to anions, cations or residual chlorine, while AA diluted in tap water (pH 3) or deionized water was more stable.

2. Amino acids: The HS-containing amino acid (Cysteine) was quite effective, presumably because this reducing agent is effective in suppressing oxidation of AA. In a previous paper (9), dehydroascorbic acid (DHAA) was reduced to AA in a Cys solution at pH 6-7.5. We subsequently applied this method to determination of AA in human plasma (10).

The effects of Ala and Cys in aqueous solutions pH 5.5-6 are shown. Standard AA diluted in phosphate buffer (pH 6) was not always stable (13). Generally, food sample solutions, except for orange juices, also show very similar pH (pH ca. 6), while human urine has a pH of 5-8. Amino acids chelate some metal ions such as iron and copper, which attack AA, suggesting that AA in the presence of amino acids is protected from oxidation by metal ions.

3. Polyphenol: Electrochemically active tannin, which is present in Japanese tea, also improved the stability of AA.

4. AA-free foods: Vinegar and honey also stabilized AA well, but sugar did not. Ethanol afforded greater stability of AA than tap water alone.

5. Urinary compounds: Electrochemically active uric acid and urea also enhanced the stability of AA.

6. Organic acids: Citric acid protected AA from degradation. It chelates metal ions and the aqueous solution gives a pH of ca. 2.5 without need for further adjustment.

These results strongly indicate that other compounds present in foods or urine contribute to the stability of AA.

Citric acid, Cys, uric acid and urea were the most effective additives.

Citric acid was chosen in the mobile phase and sample medium because it gives pH 2.5 in solution without further adjustment, because it chelates some metal ions, and because it is not electroactive at low anodic potentials. Cys, uric acid and urea were not suitable for the present study because Cys and uric acid are electrochemically active.

Applied Potential and Background Current

Next, we examined the background current (nA) developed by the mobile phase (20 mM citric acid, pH 2.5) and the relationship between applied potential and sensitivity of AA (***T2***). The current (peak height) at each applied potential was divided by the current at the most positive potential to obtain the relative peak height.

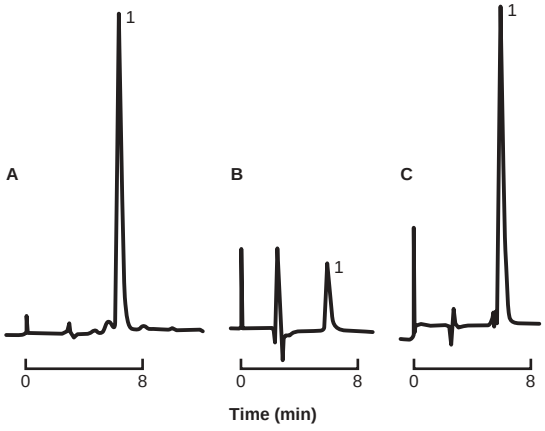
The detector gave an increasing response up to +700 mV, versus an Ag/AgCl reference electrode for AA

T1. Effect of some co-existing compounds (20 µM) on the stability of standard AA (2.5 µM) diluted with tap water stored in a brown flask at 25 ºC.

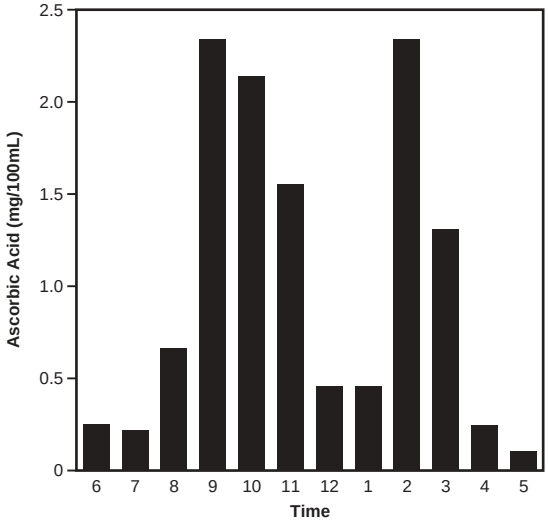
Compounds	Recovery (%) [1] 0	30	60 (min)
Tap water	63.8	0	0
Tap water (pH 3)	90.6	85.5	81.2
Deionized water	100	84.6	70.1
Citric acid	100	100	100
Ala	100	100	94.1
Cys	100	100	100
Tannin	100	100	93.1
Sodium ureate	100	100	100
Urea	100	100	100
Sugar [2]	100	100	82.5
Honey [2]	100	100	92.1
Ethanol	100	85.6	80.1

1) RSD (n=3) was 0 to 3.8%.
2) 300 mg/100 mL

F1. Chromatograms of AA from human urine, sweat and tears diluted with mobile phase. Potential set at +400 mV versus an Ag/AgCl reference electrode. (A= human urine, B= human sweat, C= human tears) LC was carried out on a 15 X 0.46 cm I.D. column of Inertsil ODS-3 (5µm) using 20 mM citric acid (pH 2.5) as a mobile phase at a flow-rate of 0.6 mL/min under ambient conditions. Peak 1 = AA



F2. Relationship between healthy human urine AA concentration and post-eating times over the 12 h period 6 a.m. - 6 p.m. (waking time = 6 a.m., breakfast = 7 a.m., lunch = 12 p.m.) (No teas and foods were taken at 10 a.m. and 3 p.m.)



and then a constant response (100%) up to 1000 mV. When the applied potential was set at +900 mV versus an Ag/AgCl reference electrode, not only AA, but also other electrochemically active compounds such as UA, cysteine and tyrosine (9,10) were detected and dilution of sample solution was necessary due to their higher sensitivity. Furthermore, dilution was accompanied by a poor RSD. In view of this, an applied potential +400 mV versus an Ag/AgCl was selected for routine determination of AA.

A comparison of the background current exhibited by a mobile phase from the literature (100 mM KH_2PO4 with 1 mM EDTA, pH 3.0, [1, 11]) to that of the present proposed mobile phase indicated that the former was not usable above 700 mV because the background current exceeded 100 nA. A comparison of 100 mM KH_2PO_4 with EDTA and EDTA-free showed that the former gave the higher background current. This suggests that EDTA is electrochemically active and is contributing to the background.

From the above, the naturally-occurring citric acid was the basis of a suitable mobile phase for determination of AA, because it chelates some metal ions and gives a suitable pH (ca. 2.5) without further adjustment. In addition, it stabilizes AA and shows lower background current.

Chromatography

Effort was then focused on the chromatography of AA from various biological fluids (***F1***) diluted with mobile phase.

Highly selective and rapid detection of AA (retention time: ca. 6.4 min) in the presence of a variety of other compounds is possible at a potential +400 mV versus an Ag/AgCl reference electrode. This allows dilution and injection without need for sample clean-up. The limit of detection from ***F1a*** was ca. 0.1 ng per injection (20 µL) at a signal-to-noise ratio of 3:1.

Determination of AA

The calibration curve for AA was constructed by plotting peak height against amount injected. The response was linear over the range 0.1 to 16 ng on column (Y = 1.053 X -0.026, Y = peak height, X = amount of AA in ng, r^2 = 1.000).

A known amount of AA was added to Japanese tea or human urine and overall recoveries were estimated by the standard addition method. As listed in ***T3***, AA recovery was over 90%. The relative standard deviations (RSD) in Japanese tea and human urine were 2.9% (n = 5) and 3.3% (n = 5), respectively. Between-day RSD (5 days) was ca. 3.7%.

T4 shows determination of AA in food samples. AA was not detected in teas, coffee, honey, freshly prepared grape juices and grated carrot. AA was determined in other food samples with RSDs of 2.5 to 3.5%.

T5 shows sampling times and analytical data for AA, including previously reported results for AA in human plasma (10).

Although the sampling method was different, average levels of AA at 10 a.m. of human plasma, urine and tears were 8.4 (n=4), 26.7 (n=2) and 13.2 (n=2) µg/mL, respectively. AA concentration ratios for plasma, urine and tears were approximately 1:3.2:1.6. The urine level of AA was twice as high as that of tears. There was good agreement for AA in tears between literature values (11.4 µg/mL) (4) and our own (13.2 µg/mL).

We examined the variation of AA in healthy human urine over a 12 h period from 6 a.m. to 6 p.m. to gauge the relationship between AA urine concentration and time after food intake (***F2***). AA urine level was lower soon after waking and before breakfast, was higher 2 to 3 h after having breakfast or lunch, and then decreased slowly with time.

Conclusion

It is possible to replace MPA and previously reported mobile phases, with their need for further pH adjustment (12-14), with the proposed mobile phase. This demonstrated pre-analysis stabilization of AA in foods and biological fluid. It is an advantage for routine work that we do not need to prepare separate solutions for sample preparation and the LC/EC mobile phase. We chose citric acid for the present purpose after examination of AA stability in samples.

A comparison of recovery and RSD obtained by the proposed method and published methods demonstrated that

T2. Comparison of the background current of each mobile phase and the effect of applied potential on the peak currents of AA and UA.

Applied potential (mV)	Background current (nA) 20 mM Citric acid (pH 2.5)	100 mM KH_2PO_4 (pH 3.0)	100 mM KH_2PO_4 with 1mM EDTA (pH 3.0) (9)	20 mM MSG[3] (pH 2.1)	RPH[2] AA	RPH[2] UA
100	0	0	0	0	3.7	0
200	0	0	1.9	0	18.6	0
300	5.5	0.8	2.1	2.4	37.3	0
400	14.6	6.6	21.4	5.2	81.1	0
500	25.2	10.4	126	21.7	85.7	0
600	71.0	20.6	154	30.7	91.2	2.1
700	78.5	24.7	-[1]	39.5	100	50.5
800	83.2	29.5	-	48.5	100	82.4
900	124	51.7	-	82.1	100	85.7
1000	275	108	-	-	100	100

1) not measured due to higher background current (over 1000 nA)
2) relative peak height
3) MSG, monosodium L-glutamate

T3. Recoveries of AA added to Japanese tea and healthy human urine.

	Added	Found	Recovery	Recovery (%)
Japanese tea	0[1]	4.14[2]	-	-
	1	5.05	.91	91
	2	6.01	1.87	93.5
	4	7.89	3.75	93.8
Healthy human urine	0[3]	31.5[4]	-	-
	10	40.7	9.2	92.0
	20	49.9	18.4	92.0
	30	59.3	27.8	92.3

1) mg/g
2) RSD : 2.9% (n=5) with no addition of AA
3) µg/mL
4) RSD : 3.3% (n=5) with no addition of AA

T4. Content of AA in foods.

		AA	RSD[1]
Japanese tea (mg/g)	A	4.14	2.9%
	B	3.24	3.1
	C	2.23	2.8
Tea (mg/g)	A	-[4]	-
	B	-	-
Coffee (mg/g)		-	-
Honey (mg/g)		-	-
Potato (boiled) (mg/g)	A	0.05	3.5
	B	0.06	3.3
Sweet potato (baked) (mg/g)		0.02	3.5
Cow's milk (mg/100 mL)		0.7	3.3
Infant milk (mg/100 g)		52.2	2.8
Juice (mg/100 mL)			
lemon[2]		40.2	2.6
tangerine[2]		39.6	3.1
grape[2]	A	-	-
	B	-	-
orange[3]		26.2	2.5
apple[3]		25.9	2.9
grated Japanese radish		12.1	3.1
grated carrot		0	-

1) n=5
2) freshly prepared prior to use
3) commercially available
4) not detected

T5. Content of AA in healthy human plasma, urine, tears and sweat.

Samples		Sampling time	AA (µg/mL)
Plasma (10)	A	a.m. 10	7.79
	B	10	8.88
	C	10	7.95
	D	10	9.17
Urine	A	a.m. 10	21.9
	B	10	31.5
	C	p.m. 3	41.3
Tears	A	a.m. 10	12.1
	B	10	14.3
Sweat[1]	A	p.m. 1	0.11
	B	1	0.12

1) Sweat was collected in a brown flask within 10 min from the subject, who had run for ca. 20 min before lunch.

T6. Comparison of recovery and RSD obtained by proposed method and published methods.

Proposed Method			
Sample	Japanese tea		Human urine
Sample medium	mobile phase		mobile phase
Mobile phase	20 mM citric acid (pH 2.5)		20 mM citric acid (pH 2.5)
Injection volume	20 µl		20 µl
AA concentration	100 ~ 800 ng/mL		100 ~ 800 ng/mL
Applied potential	400 mV		400 mV
Recovery (RSD)	over 90% (2.9%)		over 90% (3.3%)
Published Method			
	(9)	**(10)**	**(13)**
Sample	orange juice	human plasma	athlete's food
Sample medium	deionized water	10 mM KH_2PO_4 (pH 6.8 with Cys)	mobile phase
Mobile phase	100 mM KH_2PO_4 (pH 3) with EDTA	100 mM KH_2PO_4 (pH 3) with EDTA	20 mM MSG (pH 2.1)
Injection volume	20 µL	20 µL	5 µL
AA concentration	1-10 µg/mL	1-10 µg/mL	0.25-2.5µg/mL
Applied potential	300 mV	300 mV	400 mV
Recovery (RSD)	over 90% (3.2%)	over 90% (2.8%)	over 90% (2.5%)

the former's recovery and RSD were over 90% and 2.9% and 3.3%, and the latter's were over 90% and 3.2%, 2.8% and 2.5%, respectively (***T6***). So there were no significant differences in recovery and RSD between the proposed and published methods, despite different samples, sample media, mobile phases, applied potentials, injection volumes and AA concentrations.

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