

# Ascorbic Acid Concentration and Total Antioxidant Activity of Human Tear Fluid Measured Using the FRASC Assay

Camus Kar Man Choy,<sup>1</sup> Iris Frances Forster Benzie,<sup>2</sup> and Pauline Cho<sup>1</sup>

**PURPOSE.** To evaluate a novel method (FRASC) for total ferric reducing (antioxidant) activity and ascorbic acid concentration applied to human tears, to investigate the stability of ascorbic acid, and to determine the antioxidant status of human reflex tears.

**METHODS.** Linearity, sensitivity, and precision of FRASC and ascorbic acid loss during 7 days' storage were assessed; total antioxidant activity and ascorbic acid and uric acid concentrations of reflex tears from 47 healthy subjects were measured.

**RESULTS.** FRASC has good precision, linearity, and sensitivity. Ascorbic acid is stable for at least 7 days at moderately acidic pH (pH 3.6) and low temperature. Total antioxidant activity and ascorbic acid and uric acid concentrations (mean  $\pm$  SD) in reflex tears were  $409 \pm 162$ ,  $23 \pm 9.6$ , and  $68 \pm 46$   $\mu$ M, respectively. Ascorbic acid and uric acid constituted around half the total antioxidant activity measured. There was a significant correlation between uric acid and total antioxidant activity ( $r = 0.754$ ;  $P < 0.0001$ ). Men had significantly ( $P = 0.0045$ ) higher tear ascorbic acid concentrations than women.

**CONCLUSIONS.** FRASC is suitable for measuring total antioxidant activity and ascorbic acid in human tears. Further clinical study is needed to investigate the male-female difference seen, to characterize the remaining 50% antioxidant activity, and to investigate the effects of environmental conditions, antioxidant supplementation, age, and ocular disease on tear antioxidant status. (*Invest Ophthalmol Vis Sci.* 2000;41:3293-3298)

The ability of antioxidants to scavenge reactive oxygen species (ROS) is important to protect tissues from light-induced oxidative damage.<sup>1,2</sup> This is particularly true of the ocular tissues because of their exposure to light, which causes production of ROS in situ.<sup>3-8</sup> Uric acid, an endogenous purine breakdown product that has been suggested to be an important antioxidant,<sup>2,6,8</sup> has been detected in tear fluid.<sup>9</sup> Ascorbic acid (vitamin C), an essential diet-derived antioxidant, is found in a high concentration in the aqueous humor,<sup>10</sup> but there is no agreement regarding its concentration in human tears.<sup>3,9,11,12</sup> Ascorbic acid is known to be destroyed rapidly in blood plasma *ex vivo*,<sup>13</sup> but its stability in tears has not been studied. In addition, little is known about the antioxidant profile or the "total antioxidant activity" of tears. This is owing to the small volume of tears that can be obtained for testing and to the fact that most analytical procedures that might be used for investigation of the composition of tears are not sensitive enough to give reliable results.<sup>9,14</sup> Speedy, sensitive, and specific methods suitable for use with small volumes are needed to

study the antioxidant composition of human tears. This information would be useful to study the effects of, for example, diet, contact lens wear, advancing age, and various environmental agents on ocular defense against ROS and may help in the understanding and prevention of ocular disease.

The gold standard method of measuring ascorbic acid is by high-performance liquid chromatography (HPLC).<sup>15</sup> However, this method requires highly specialized equipment and a high level of technical skill. In addition, HPLC is a time-consuming "one-by-one" type of analysis, which can be problematic when an unstable analyte is being measured. Furthermore, HPLC cannot be used to measure the total antioxidant activity of biological fluids.

An antioxidant can be broadly defined as any substance that delays or inhibits oxidative damage to a target molecule.<sup>2,16</sup> The measurement of total antioxidant activity of a biological fluid is a composite measurement of the combined effects of individual scavenging antioxidants within the sample and may provide insight into the overall prooxidant-antioxidant balance.<sup>2,17</sup> Several methods of measuring total antioxidant activity are available and have been used with blood plasma.<sup>18-20</sup> In this study we used the ferric reducing antioxidant power (FRAP) assay.<sup>20,21</sup> This fast and relatively simple method has been used for plasma but not, to date, for tear fluid. In addition, a recent modification of the FRAP assay, known as FRASC,<sup>21,22</sup> enables the simultaneous measurement of total antioxidant activity and ascorbic acid concentration in the sample. Unlike HPLC, the FRASC assay takes only a few minutes, is relatively inexpensive, and provides two indices of antioxidant status. This makes FRASC a potentially valuable

---

From the Departments of <sup>1</sup>Optometry and Radiography and <sup>2</sup>Nursing and Health Sciences, The Hong Kong Polytechnic University, Kowloon, Hong Kong SAR, China.

Supported by Research Grant GV752 from the Hong Kong Polytechnic University.

Submitted for publication February 24, 2000; revised May 5, 2000; accepted May 22, 2000.

Commercial relationships policy: N.

Corresponding author: Iris Frances Forster Benzie, Department of Nursing and Health Sciences, The Hong Kong Polytechnic University, Kowloon, Hong Kong. [hsbenzie@polyu.edu.hk](mailto:hsbenzie@polyu.edu.hk)

tool for use with fluids, such as tears, where both measurements are desirable and where sample volumes are small.

The speed of FRASC is of particular benefit in measuring unstable antioxidants, such as ascorbic acid, which are destroyed rapidly *ex vivo*. Ascorbic acid is often stabilized in biological fluids *ex vivo* by the addition of strong acid, such as metaphosphoric acid.<sup>13</sup> However, this method of stabilization cannot be used for samples to be analyzed by FRASC, because the enzyme used (ascorbic oxidase) in the assay would be denatured. It was of interest, therefore, to investigate whether ascorbic acid could be adequately stabilized in solution at pH 3.6, the pH of the FRASC reaction.

The aims of this study, therefore, were to evaluate FRASC for the simultaneous measurement of total antioxidant activity and ascorbic acid concentration in human tears, to investigate the stability of ascorbic acid at a pH compatible with the use of FRASC as a measuring tool, and to investigate the antioxidant status, based on total antioxidant activity and ascorbic acid and uric acid measurements, of tear fluid from healthy eyes.

## METHODS

Experiments were performed to evaluate the FRASC assay, in terms of linearity, precision, and detection limit, for use with tear fluid, to investigate the stability of ascorbic acid at pH 3.6, and to determine the ascorbic acid and uric acid concentrations and the total antioxidant activity (as the FRAP value) in tear fluid from healthy young adults ( $n = 47$ ).

Ascorbic acid standards (between 0 and 100  $\mu\text{M}$ ) were prepared in 300 mM acetate buffer (pH 3.6) and, separately, in aged tears. These solutions were used immediately after preparation. Aged tears refers to pooled tear fluid from healthy subjects, which was stored at room temperature for 3 days before use. This aged tear fluid contained no native ascorbic acid (results not shown). Different amounts of ascorbic acid in acetate buffer (pH 3.6) were added to the aged tears to produce tear-based solutions of known ascorbic acid concentration at pH 3.6. To assess linearity, one set of tear-based ascorbic acid standards (0, 2.5, 12.5, 25.0, and 50.0  $\mu\text{M}$ ) was prepared and measured in duplicate, and nine sets of ascorbic acid standards in acetate buffer (0, 5, 25, 50, and 100  $\mu\text{M}$ ) were prepared and each set was measured singly. The limit of detection for ascorbic acid in the FRASC assay was assessed by calculating the mean + 3 SD of the absorbance readings of a sample containing no ascorbic acid (distilled water) measured 10 times. Precision was assessed by nine measurements of one set of standards run in parallel (in-run), and of six sets of standards run on separate days (between-run). To assess stability in moderately acidic medium, solutions of ascorbic acid (pH 3.6) of known concentration were divided into aliquots and stored at  $-70^\circ\text{C}$ ; at 1, 2, 3, 4, 5, and 7 days after preparation, an aliquot of each solution was thawed and the ascorbic acid concentration was measured and compared with that of the freshly prepared (at day 0) solution.

For tear analysis, 47 young adults (29 men, 18 women; mean  $\pm$  SD age,  $23 \pm 3$  years) were recruited. Subjects were apparently healthy nonsmokers who were not on medication or taking vitamin supplements. From each subject approximately 120  $\mu\text{l}$  of tears, induced by the yawn reflex, was collected using 20  $\mu\text{l}$  disposable capillary tubes (Drummond Scientific, Broomall, PA) and according to the procedure of Callender and Morrison.<sup>23</sup>

To determine whether there was any antioxidant or interfering contamination of the tear sample due to contact with the capillary tube, freshly prepared ascorbic acid standards in acetate buffer were measured with and without passage through capillary tubes, which showed no difference (results not shown).

Reagents and equipment used for the FRASC assay for total antioxidant power and ascorbic acid concentration measurement were as described in detail elsewhere,<sup>14,20-22</sup> with the use of a Cobas Fara centrifugal analyzer (Roche Diagnostics, Basel, Switzerland). In brief, the FRASC method uses the ability of antioxidants to reduce an  $\text{Fe}^{\text{III}}$ -TPTZ complex to its blue-colored  $\text{Fe}^{\text{II}}$  form. The change in absorbance at 593 nm ( $\Delta A_{593\text{nm}}$ ) after a reaction time of 4 minutes is due to the combined activity of all the reacting antioxidants present in the sample (i.e., the total ferric reducing [antioxidant] power, or FRAP value).<sup>20</sup> Ascorbic acid is selectively destroyed in one of a pair of samples by ascorbic oxidase, and the difference between paired samples in terms of their absorbances at 593 nm after a 1-minute reaction time is due to ascorbic acid.<sup>20,21</sup> FRASC reagents were as follows: 300 mM acetate buffer (pH 3.6) was prepared by dissolving 3.1 g sodium acetate trihydrate (Riedel-de Haen, Hannover, Germany) in distilled water, with 16 ml glacial acetic acid (BDH Laboratory Supplies, Poole, England) added; this was made up to one liter with distilled water; a 10 mM TPTZ (2,4,6 tripyridyl-s-triazine; Fluka Chemicals, Buchs, Switzerland) solution in 40 mM HCl (BDH) and a 20 mM  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  (BDH) solution in distilled water were prepared. Working FRASC reagent was prepared freshly as required by mixing 25 ml acetate buffer with 2.5 ml of TPTZ solution and 2.5 ml of ferric chloride solution. A 4 IU/ml solution of ascorbic oxidase (EC 1.10.3.3 from Sigma Chemical, St. Louis, MO) was prepared in distilled water, divided into aliquots, and stored at  $-70^\circ\text{C}$  until use. Freshly prepared aqueous solutions of  $\text{Fe}^{\text{II}}$  (1000  $\mu\text{M}$  from  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ; Riedel-de Haen) and ascorbic acid (5, 25, 50, and 100  $\mu\text{M}$  from extra pure crystals; Merck, Darmstadt, Germany) were used for calibration of the assay.

To prepare the samples for FRASC and uric acid analysis, 60  $\mu\text{l}$  of each sample (tears, ascorbic acid test solutions, and calibrators) was mixed with 20  $\mu\text{l}$  300 mM acetate buffer in each of two sample cups. To one of the paired cups, 32  $\mu\text{l}$  of ascorbic oxidase solution was added; 32  $\mu\text{l}$  of distilled water was added to the other paired cup. Calibrators were also treated in pairs. After mixing, the paired ascorbic oxidase-diluted ("ao") and water-diluted ("-ao") samples were loaded onto the analyzer for automated measurement. Calculation of FRASC results was performed from absorbance readings as follows<sup>21,22</sup>:

Using the water-diluted samples, the FRAP ( $\mu\text{M}$ ) value =

$$\frac{0-4 \text{ min } \Delta A_{593\text{nm}} \text{ of test sample}}{0-4 \text{ min } \Delta A_{593\text{nm}} \text{ of standard}} \times [\text{standard}] (\mu\text{M})$$

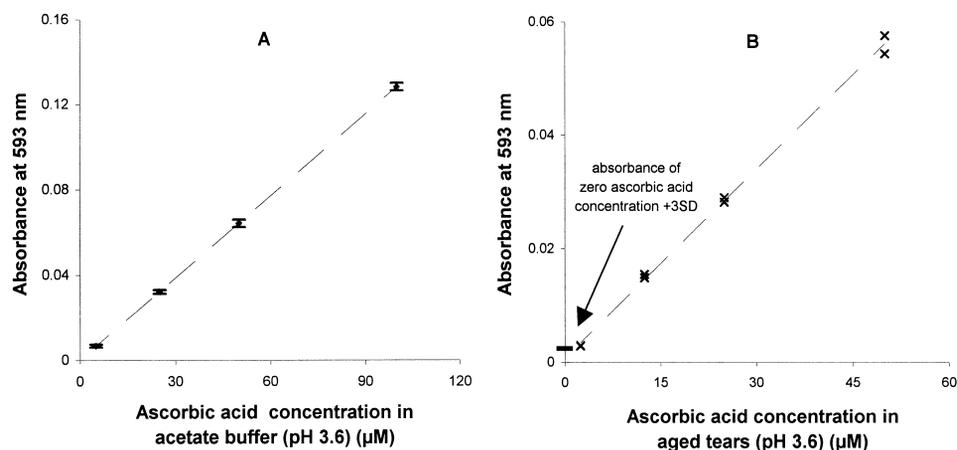
Using the paired water- (-ao) and ascorbic oxidase-diluted (+ao) samples, the ascorbic acid concentration was calculated as follows:

0-1 min ascorbic acid-related  $\Delta A_{593 \text{ nm}}$

$$= (0-1 \text{ min } \Delta A_{593 \text{ nm}} \text{ sample } -\text{ao}) \quad (1)$$

$$- (0-1 \text{ min } \Delta A_{593 \text{ nm}} \text{ sample } +\text{ao})$$

**FIGURE 1.** Linearity of the FRASC method for ascorbic acid, in acetate buffer (A), pH 3.6. Each point represents the mean of nine measurements with  $\pm 1$  SD error bars shown and in aged tears (B) adjusted to pH 3.6, measured in duplicate, with both measurements shown. Results showed good linearity over the range tested. The limit of detection, based on mean + 3 SD of 10 readings of distilled water as test solution, was 2.5  $\mu\text{M}$ .



ascorbic acid concentration ( $\mu\text{M}$ ) =

$$\frac{0-1 \text{ min ascorbic acid-related } \Delta A_{593 \text{ nm}} \text{ of test sample}}{0-1 \text{ min ascorbic acid-related } \Delta A_{593 \text{ nm}} \text{ of standard}} \quad (2)$$

$$\times [\text{standard}] (\mu\text{M})$$

Uric acid was measured using a commercially available uricase/PAP kit (Unimate 7; Roche Diagnostics) on a Cobas Fara and following the manufacturer's instructions.

This study was approved by the Human Subjects Ethics Subcommittee of the Hong Kong Polytechnic University. All subjects gave their informed consent to take part in the study, and all procedures involving human subjects complied with the Declaration of Helsinki, as revised in 1989.

For statistical analysis, the unpaired *t*-test was used to detect differences between males and females; Pearson's correlation was used to investigate relationships, and repeated measures ANOVA test was used to investigate stability.

## RESULTS

FRASC showed good linearity and sensitivity (Fig. 1), with a detection limit of 2.5  $\mu\text{M}$  ascorbic acid. Precision was also good: In-run and between-run coefficients of variation were, respectively,  $<3.0\%$  ( $n = 9$ ) and  $\leq 3.0\%$  ( $n = 6$ ) at between 5 and 100  $\mu\text{M}$  ascorbic acid and  $<2.0\%$  for both at between 10 and 200  $\mu\text{M}$  FRAP values. No decrease was seen in ascorbic acid stored in acetate buffer (pH 3.6) and low temperature ( $-70^\circ\text{C}$ ) for up to 7 days (Fig. 2). Mean  $\pm$  SD ascorbic acid and uric acid concentrations and the total antioxidant activity (as the FRAP value) of tears were, respectively,  $23 \pm 9.6$ ,  $68 \pm 46$ , and  $409 \pm 162 \mu\text{M}$ , showing a wide interindividual variation. No significant differences were found between men and women in terms of total antioxidant activity or uric acid in tears; however, men had significantly higher ( $P = 0.0045$ ) ascorbic acid concentrations (Table 1). There was a significant correlation ( $r = 0.754$ ;  $P < 0.0001$ ) between the uric acid concentration and the FRAP value of human tears, but no significant correlation was seen between ascorbic acid concentration and the FRAP value ( $r = 0.0656$ ;  $P = 0.661$ ).

## DISCUSSION

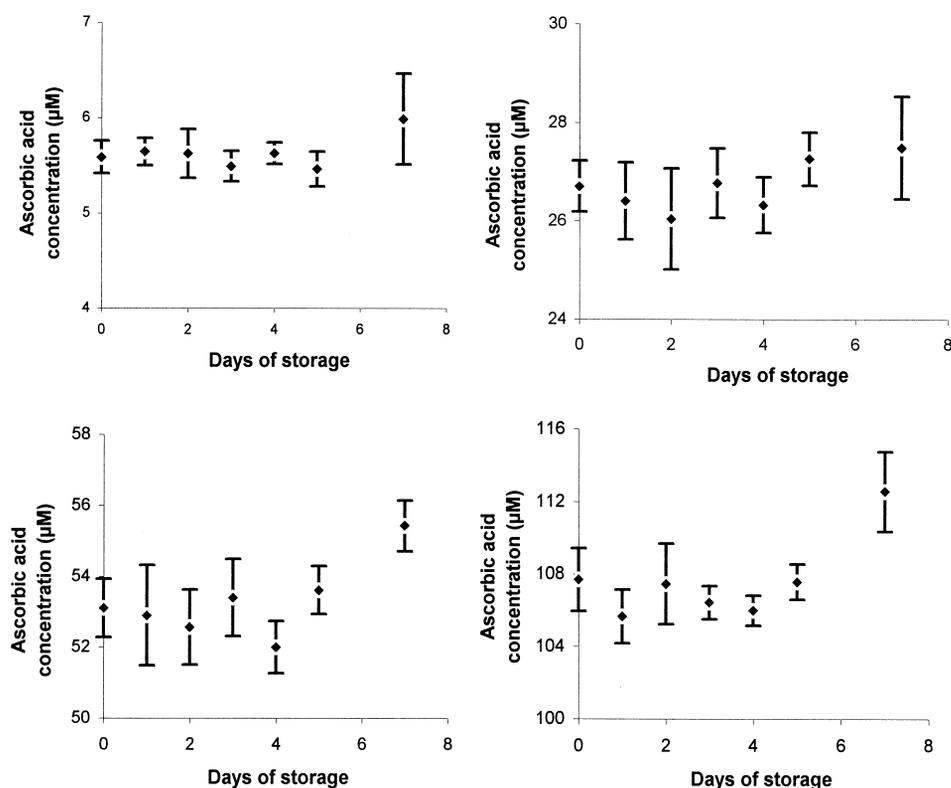
This study has shown that the FRASC assay<sup>21,22</sup> is fast, linear, sensitive, and precise enough to measure ascorbic acid con-

centration and total antioxidant activity in human tear fluid. The use of a moderately acidic medium and storage at low temperature was found to stabilize ascorbic acid in aqueous solution for at least 7 days. Results also showed that ascorbic acid contributes around 11%, and uric acid around 33%, of the total antioxidant activity (as the FRAP value) of human reflex tears.

To date there are no data on overall antioxidant status, or total antioxidant activity, of tear fluid. The antioxidant status of tear fluid is of interest because tears are the first barrier protecting the cornea against oxidative damage from radiation, atmospheric oxygen, and toxic chemicals.<sup>2,8,9,11</sup> In addition to defense, antioxidants may have a role in modulating wound healing and inflammatory responses in the cornea and in improving tear stability.<sup>4,24-28</sup> This has implications for corneal health, particularly in contact lens wearers.<sup>4,24-30</sup> In the present study, the average total antioxidant activity (as the FRAP value) of human reflex tears from apparently healthy young subjects was 409  $\mu\text{M}$ , but there was wide interindividual variation. In plasma, uric acid and ascorbic acid together contribute around 80% of the total antioxidant activity, which is around 1000  $\mu\text{M}$ , the rest being largely vitamin E, bilirubin, and protein.<sup>18-20,31</sup> In tears, uric acid and ascorbic acid account for around half the total antioxidant activity. What constitutes the other half remains to be determined; however, tear fluid is unlikely to contain significant amounts of either the lipid soluble vitamin E or bilirubin (which has an intense yellow color) and has a very low protein content.<sup>32</sup> Low levels of cysteine and tyrosine have been detected in tear fluid,<sup>9</sup> and these may contribute to the antioxidant activity of tears. There are likely to be other, as yet undetected, antioxidants, and further study is needed to characterize these, to investigate the effects of diet, and to explore the relationship between plasma and tear antioxidant status.

To date there has been only one published report of uric acid levels in human tears.<sup>9</sup> Uric acid is an endogenous compound and has been suggested to be an important physiological antioxidant.<sup>2,8</sup> However, elevated plasma uric acid is associated with coronary heart disease, diabetes, and renal failure.<sup>33,34</sup> Therefore, although uric acid may make a significant contribution to the total antioxidant activity of biological fluids, including plasma and tears, increased levels are not desirable because these indicate disease rather than health.

The results of this present study showed mean  $\pm$  SD ascorbic acid concentration of  $23 \pm 9.6 \mu\text{M}$  in reflex tears from



**FIGURE 2.** Stability of ascorbic acid. Aqueous solutions (pH 3.6) of four initial concentrations of ascorbic acid were stored at  $-70^{\circ}\text{C}$  for up to 7 days. Results showed no loss of ascorbic acid over this time. Each point represents the mean  $\pm$  1 SD of nine measurements.

47 subjects and that men had significantly higher levels than women. This is the first report of a male-female difference in tear ascorbic acid concentrations and is interesting, because men have not been found to have higher ascorbic acid concentrations in plasma.<sup>31</sup> Previously published data on tear ascorbic acid are conflicting and sparse<sup>3,9,11,12</sup> but generally showed higher concentrations than those found in the present study (see Table 2).

This may be because of the different methods of tear collection and stimulation used. In our study and that of Kuzenga et al.,<sup>3</sup> glass capillary tubes were used for collection, whereas Schirmer strips were used in other studies.<sup>9,11,12</sup> Schirmer strips are invasive and the volume collected is very small and difficult to measure accurately.<sup>35</sup> Evaporation of water from the small tear sample captured may significantly increase the apparent solute concentration. Furthermore, it has been reported that the use of Schirmer strips is associated with elevated tear plasmin concentrations,<sup>35</sup> indicating that cells on the conjunctival surface are damaged. Vascular fragility during irritation by the presence of the Schirmer strip in the lower cul-de-sac of the eye and injuries to the conjunctival surface may change the composition of the tears collected. Transudation of vascular fluid, leakage from damaged cells at the site of collection onto the Schirmer strip, or both could lead to a significant increase in ascorbic acid and uric acid concentrations of the tear fluid collected. Furthermore, corneal epithelial cells have very high concentrations of ascorbic acid.<sup>36,37</sup> Damage to these cells or the presence of contaminating corneal cells in tears collected by Schirmer strips will undoubtedly increase tear ascorbic acid concentration. However, the capillary tube used for tear collection is much less invasive than Schirmer strip. A small disposable glass capillary tube is placed just above the lower tear meniscus of the outer canthus, and

with care minimal contact between the tip of the capillary tube and the globe can be achieved.

The tear film protects the cornea and is a mixture of basal tears and reflex tears.<sup>38</sup> This study has shown that stimulated tears contain significant levels of ascorbic acid and antioxidant activity, so it is likely that the tear film also contains ascorbic acid. The immediate source of this ascorbic acid is not yet clear. Dietary ascorbic acid is distributed via the blood plasma and actively accumulated in cells owing to the actions of both a passive and an active transport mechanism.<sup>2,31,36</sup> Ocular tissues are particularly rich in ascorbic acid. It is possible that intracellular ascorbic acid acts as a reservoir to maintain levels in extracellular fluids such as tears, and leakage from the ascorbic acid-rich corneal epithelial cells may be one source of tear ascorbic acid. However, it has been shown that ascorbic acid is absorbed from plasma by the secretory acinar cells, with consequent accumulation in the lacrimal gland.<sup>39</sup> Because of ascorbic acid accumulation in this gland, it is likely that reflex tears contain ascorbic acid on secretion. Furthermore, it is possible that tear fluid is a source of corneal epithelial ascorbic acid (i.e., that tears transport ascorbic acid from the plasma to

**TABLE 1.** Total Antioxidant Activity (FRAP Value), Ascorbic Acid Concentration, and Uric Acid Concentration in Human Tears

	All (n = 47)	Men (n = 29)	Women (n = 18)
Ascorbic acid ( $\mu\text{M}$ )	23 $\pm$ 9.6	26 $\pm$ 11*	18 $\pm$ 4.3
Uric acid ( $\mu\text{M}$ )	68 $\pm$ 46	63 $\pm$ 39	77 $\pm$ 55
Total antioxidant activity ( $\mu\text{M}$ )	409 $\pm$ 162	376 $\pm$ 135	463 $\pm$ 191

Values are mean  $\pm$  SD.

\* Significantly higher than female value ( $P = 0.0045$ ).

TABLE 2. Published Data: Concentrations of Ascorbic Acid and Uric Acid in Human Tears

Studies	No. of Subjects	Methods of Tear Collection	Methods of Measurement	Local Anesthetic Used for Basal Tear Collection	Stimulation Methods for Reflex Tear Collection	Results ( $\mu\text{M}$ )
Gogia et al. (1998) <sup>9</sup>	$n = 5$	Schirmer strip for 5 min	HPLC with electrochemical detector	Proparacaine hydrochloride	30% ammonium hydroxide	Basal: aa = $665 \pm 49$ ua = $328 \pm 48$ Reflex: aa = $116 \pm 16$ ua = $131 \pm 24$
Paterson et al. (1987) <sup>12</sup>	$n = 15$	Schirmer strip for $\leq 5$ min	HPLC with spectrophotometric detector set at 245 nm	Proparacaine hydrochloride	30% ammonium hydroxide	Both basal and reflex: aa = 110–1310 ua = not done
Howard et al. (1987) <sup>11</sup>	$n = 50$	Schirmer strip for 5 min	HPLC with spectrophotometric detector set at 245 nm	Not mentioned	Not mentioned	aa = $65 \pm 34$ ua = not done
Kuizenga et al. (1987) <sup>3</sup>	$n = 8$	Capillary glass tube	Colorimetric method (with detection limit of $60 \mu\text{M}$ )	None	A jet of pressurized air and onion fumes	aa = undetectable ua = not done
Current study	$n = 47$	Capillary glass tube	FRASC assay for aa measurement and Uricase/PAP kit for ua measurement	None	Yawning	aa = $23 \pm 9.6$ ua = $68 \pm 46$

Ascorbic acid, aa; uric acid, ua.

the external ocular tissues). This mirrors the situation of tears as a source of other nutrients, such as oxygen and glucose, for the avascular cornea.

In conclusion, this study evaluated, in analytical terms, the FRASC assay for the measurement of total antioxidant activity and ascorbic acid in human tears. FRASC is a modified version of the ferric reducing (antioxidant) power (FRAP) assay and is an objective and reproducible measuring tool to assess the combined in vitro ability of scavenging, electron donating antioxidants in biological samples. However, as with all in vitro tools the clinical utility of the results obtained can only be determined by extensive clinical study. Results of this study have validated the use of the FRASC assay for measuring total antioxidant activity and ascorbic acid in human tears. Results also indicate that uric acid and ascorbic acid constitute around half the total antioxidant activity (as the FRAP value) of human reflex tears and that uric acid, but not ascorbic acid, showed a significant correlation with the total antioxidant activity. This is likely to be because of the lower concentration of ascorbic acid in tears. However, unlike the situation with uric acid, it may be possible to significantly increase the ascorbic acid content of tears by increasing the dietary intake, and this is worth further study. In addition, the data collected provide a baseline to support future clinical study to investigate the male-female difference seen here, to characterize the as yet unidentified antioxidants in tears, and to investigate the effects of environmental conditions, antioxidant supplementation, aging, and ocular disease on the antioxidant status of human tears.

### Acknowledgment

The authors thank Savio Yim Tong Szeto for his expert technical support.

### References

- Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. *Proc Natl Acad Sci.* 1993;90:7915–7922.
- Halliwell B, Gutteridge JMC. *Free Radicals in Biology and Medicine.* 3rd ed. Oxford: Oxford University Press; 1999:31–98.
- Kuizenga A, Van Haeringen NJ, Kijlstra A. Inhibition of hydroxyl radical formation by human tears. *Invest Ophthalmol Vis Sci.* 1987;28:305–313.
- Augustin AJ, Spitznas M, Kaviani N, et al. Oxidative reactions in the tear fluid of patients suffering from dry eyes. *Graefes Arch Clin Exp Ophthalmol.* 1995;233:694–698.
- Shimmura S, Suematsu M, Shimoyama M, Tsubota K, Oguchi Y, Ishimura Y. Subthreshold UV radiation-induced peroxide formation in cultured corneal epithelial cells: the protective effects of lactoferrin. *Exp Eye Res.* 1996;63:519–526.
- Leske MC, Chylack LT, He Q, et al. Antioxidant vitamins and nuclear opacities. *Ophthalmology.* 1998;105:831–836.
- Reddy VN, Giblin FJ, Lin LR, Chakrapani B. The effect of aqueous humor ascorbate on ultraviolet-B-induced DNA damage in lens epithelium. *Invest Ophthalmol Vis Sci.* 1998;39:344–350.
- Rose RC, Richer SP, Bode AM. Ocular oxidants and antioxidant protection. *Proc Soc Exp Biol Med.* 1998;217:397–407.
- Gogia R, Richer SP, Rose RC. Tear fluid content of electrochemically active components including water soluble antioxidants. *Curr Eye Res.* 1998;17:257–263.
- Taylor A, Jacques PF, Nadler D, Morrow F, Sulsky SI, Shepard D. Relationship in humans between ascorbic acid consumption and levels of total and reduced ascorbic acid in lens, aqueous humor, and plasma. *Curr Eye Res.* 1991;10:751–759.
- Howard RR, Peterson T, Kastl PR. High-performance liquid chromatographic determination of ascorbic acid in human tears. *J Chromatogr.* 1987;414:434–439.
- Paterson CA, O'Rourke MC. Vitamin C levels in human tears. *Arch Ophthalmol.* 1987;105:376–377.
- Margolis SA, Duewer DL. Measurement of ascorbic acid in human plasma and serum; stability, intralaboratory repeatability, and interlaboratory reproducibility. *Clin Chem.* 1996;42:1257–1262.

14. Benzie IFF. An automated, specific, spectrophotometric method for measuring ascorbic acid in plasma (EFTSA). *Clin Biochem.* 1996;29:111-116.
15. Rose R, Nahrwold DL. Quantitative analysis of ascorbic acid and dehydroascorbic acid by high-performance liquid chromatography. *Anal Biochem.* 1981;114:140-145.
16. Halliwell B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? *Lancet.* 1994;344:721-724.
17. Strain JJ, Benzie IFF. Diet and antioxidant defence. In: Sadler M, Strain JJ, Cabellero B, eds. *The Encyclopedia of Human Nutrition.* 1st ed. London: Academic Press; 1999:95-105.
18. Whitehead TP, Thorpe GHG, Maxwell SRJ. Enhanced chemiluminescent assay for antioxidant capacity in biological fluids. *Anal Chim Acta.* 1992;266:265-277.
19. Miller NJ, Rice-Evans CA. Spectrophotometric determination of antioxidant activity. *Redox Rep.* 1996;2:161-171.
20. Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem.* 1996;239:70-76.
21. Benzie IFF, Strain JJ. Simultaneous automated measurement of total 'antioxidant' (reducing) capacity and ascorbic acid concentration. *Redox Rep.* 1997;3:233-238.
22. Benzie IFF, Strain JJ. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. In: Packer L, ed. *Methods in Enzymology: Oxidants and Antioxidants Part A.* 1st ed. Vol. 299. London: Academic Press Limited; 1999:15-27.
23. Callender M, Morrison PE. A quantitative study of human tear proteins before and after adaption to non-flexible contact lenses. *Am J Optom Physiol Optics.* 1974;51:939-945.
24. Wright P. The chemically injured eye. *Trans Ophthalmol Soc UK.* 1982;102:85-87.
25. McGahan MC. Ascorbic acid levels in aqueous and vitreous humors of the rabbit: effects of inflammation and ceruloplasmin. *Exp Eye Res.* 1985;41:291-298.
26. Williams RN, Paterson CA. Modulation of corneal lipoxygenase by ascorbic acid. *Exp Eye Res.* 1986;43:7-13.
27. Williams RN, Paterson CA. A protective role for ascorbic acid during inflammatory episodes in the eye. *Exp Eye Res.* 1986;42:211-218.
28. Patel S, Plaskow J, Ferrier C. The influence of vitamins and trace element supplements on the stability of the pre-corneal tear film. *Acta Ophthalmol.* 1993;71:825-829.
29. Korb DR, Greiner JV, Glonek T. Tear film lipid layer formation: implications for contact lens wear. *Optom Vis Sci.* 1996;73:189-192.
30. Tsubai T, Murai M, Watanabe A. Dry eye: a contraindication for contact lens wear? Do tears protect the corneal surface against photo-oxidation stress? *Orbit.* 1997;16(suppl):91-99.
31. Benzie IFF, Janus ED, Strain JJ. Plasma ascorbate and vitamin E levels in Hong Kong Chinese. *Eur J Clin Nutr.* 1998;52:447-451.
32. Ng V, Cho P, To CH. Tear proteins of normal young Hong Kong-Chinese. *Graefes Arch Clin Exp Ophthalmol.* In press.
33. Benzie IFF, Strain JJ. Uric acid: friend or foe? *Redox Rep.* 1996;2: 231-234.
34. Lehto S, Niskanen L, Ronnema T, Laakso M. Serum uric acid is a strong predictor of stroke in patients with non-insulin-dependent diabetes mellitus. *Stroke.* 1998;29:635-639.
35. Van Setten GB, Stephens R, Tervo T, Salonen EM, Tarkkanen A, Vaheri A. Effects of the Schirmer test on the fibrinolytic system in the tear fluid. *Exp Eye Res.* 1990;50:135-141.
36. Reim M, Seidl M, Brucker K. Accumulation of ascorbic acid in the corneal epithelium. *Ophthalmic Res.* 1978;10:135-139.
37. Ringvold A, Anderssen E, Kjonniksen I. Distribution of ascorbate in the anterior bovine eye. *Invest Ophthalmol Vis Sci.* 2000;41:20-23.
38. Jordan A, Baum J. Basic tear flow: does it exist? *Ophthalmology.* 1980;87:920-930.
39. Dreyer R, Rose RC. Lacrimal gland uptake and metabolism of ascorbic acid. *Proc Soc Exp Biol Med.* 1993;202:212-216.