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Short Communication

Determination of ascorbic acid and uric acid in plasma by high-performance liquid chromatography

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Abstract

A simple method with excellent reproducibility has been developed for the simultaneous determination of the plasma concentration of ascorbic acid and uric acid by reversed-phase HPLC using an ion-pairing reagent with UV detection. Coefficients of variation for the concentrations of ascorbic acid and uric acid in plasma are $<2.1\%$ and $<3.5\%$ respectively.

1. Introduction

Ascorbic acid (vitamin C) is an antioxidant [1] normally found at a concentration of 30–80 μM in plasma. Uric acid, a product of purine metabolism, also has antioxidant activity [2], and occurs at around 300 μM in plasma.

Many published HPLC methods for the determination of ascorbic acid are based on electrochemical detection [3] and use cationic detergents such as octylamine and tridecyl ammonium formate, which act as ion-pairing reagents. UV detection can also be used but usually requires ascorbic acid to be derivatized. Methods not requiring derivatization of ascorbic acid often involve the use of very acidic conditions [4] or ion-pairing reagents. For a general review of methods see Pachla and Reynolds [5].

Despite the development of many methods, there are still a number of problems associated with the determination of ascorbic acid.

(i) Reproducibility is a major problem especially when using ion-pairing reagents. This may be caused by variable retention times and inadequate equilibration of the column with solvent.

(ii) Column durability decreases when using very acidic conditions.

(iii) The choice of method is dependent on the availability of detectors with cost efficiency becoming more important when measuring large numbers of samples.

The method described below was developed to overcome these problems.

2. Experimental

2.1. Chemicals

Ascorbic acid, EDTA, acetic acid, sodium hydroxide, perchloric acid (all Analar grade), metaphosphoric acid (general purpose reagent grade), acetonitrile (HPLC grade), were obtained from Merck (Poole, UK). Uric acid, homocysteine, ascorbic acid oxidase, uricase and

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myristyltrimethylammonium bromide were obtained from Sigma (Poole, UK). USP and European reference standards for ascorbic acid were supplied by Promochem (St. Albans, UK).

2.2. Chromatographic conditions

The HPLC system consisted of two pumps (Gilson 305, Anachem, Luton, Beds, UK) and a 20-μl loop volume automated sampler module (Gilson 231) which was connected to a UV detector (Gilson 116). The automatic sampler was kept at 4°C with a Flow Cooler (Techne FC-200, Anachem, Luton, Beds, UK) and Circulator (Techne C-85D). The system was monitored by a computer (Dell 433 s/L, Dell Computer Corporation, Bracknell, Berkshire, UK) with software (Gilson 714).

A Nucleosil ODS 5-μm column (Jones, Henygoed, Mid Glamorgan, UK) 25 × 0.46 cm I.D. with guard column 2 × 0.4 cm I.D. (Anachem) packed with pellicular C_{18} reversed-phase packing 30–40 μm (Perisorb RP.18, Anachem) was used for the separation.

The mobile phase was 25 m*M* myristyltrimethylammonium bromide, 0.05 *M* sodium hydroxide, 0.06 *M* acetic acid, 7.5% (v/v) acetonitrile, pH 5.5. Homocysteine at 100 mg/l and EDTA at 200 mg/l were added before use. The system was operated at ambient temperature with a flow-rate of 0.55 ml/min and UV detection at 262 nm.

2.3. Sample preparation

Heparinized whole blood was centrifuged at 2000 *g*, 4°C for 15 min and 0.6-ml aliquots of the plasma were added to an equal volume of 10% (w/v) metaphosphoric acid. The samples were immediately snap frozen and stored at −80°C. All plasma was prepared within an hour of blood collection.

For analysis, the samples were allowed to thaw at 4°C for one hour, centrifuged at 1000 *g*, 4°C for 10 min and the clear supernatants removed and aliquoted into amber vials.

2.4. Standards

Samples for standard curves were prepared in a similar way to the plasma extracts. Ascorbic acid and uric acid were made up separately in 5% (w/v) metaphosphoric acid. Standard curves covered a range of concentrations up to 200 μM for ascorbic acid and up to 500 μM for uric acid. European and USP standards for ascorbic acid were also made up as described above, divided into aliquots, and frozen at −80°C.

In addition a pooled plasma of approximately 200 ml was also prepared using the above method. For quality control three reference standards were run with each 25 unknown samples; a pooled plasma, USP ascorbic acid and a European reference ascorbic acid.

3. Results

3.1. Chromatography

The identity of the uric acid and ascorbic acid peaks was confirmed by the addition of uricase, which caused the disappearance of the uric acid peak, and ascorbic acid oxidase which caused the disappearance of the ascorbic acid peak. The peaks were very well separated (Fig. 1) in under 10 min. In one batch of 12 samples the mean retention time of ascorbic acid was 5.32 ± 0.05 min, and that of uric acid was 4.37 ± 0.03 min.

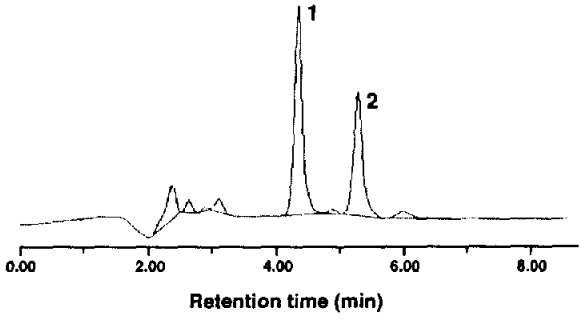

Fig. 1. Chromatogram of human plasma. Peaks: 1 = uric acid, 2 = ascorbic acid.

Over a 2-week period the mean retention times of the ascorbic acid and uric acid were 5.35 ± 0.08 min and 4.39 ± 0.05 min respectively.

3.2. Linearity

Five-point (duplicated) calibration graphs necessary for ascorbic acid standards (0–200 μM) showed good linearity, passing through the origin: $y = 3.727x + 1.786$, $r = 0.9999$. Uric acid standards (0–500 μM) also showed good linearity: $y = 1.337x + 12.558$, $r = 0.9997$.

3.3. Assay validation

Method validation was defined in terms of the intra-batch and inter-batch precision, which was established by analysing replicate pooled plasma samples. The intra-batch coefficient of variation (C.V.) for the ascorbic acid and uric acid in plasma samples are shown in Table 1.

The inter-batch precision was checked by analysing five batches on separate days over a period of 2 weeks. The C.V. for ascorbic acid was 1.0% and for uric acid 3.4%.

4. Discussion

In aqueous solutions at pH 5.5, the ascorbate ion [AH^-] predominates, since the pKa of ascorbic acid is 4.19. Similarly the urate ion also predominates, since the pKa of uric acid is 5.4 [6]. In ion-pairing chromatography the ion-pairing reagent pairs with these polar compounds to give neutral compounds which can be separated by reversed-phase chromatography.

The mechanism of the ion-pairing reagent is such that an equilibrium is set up between the free ion-pairing reagent in the mobile phase, the ion pair bonded on to the column packing and the ion-pairing reagent paired with the compound. Therefore, Dolan [7] recommended the use of a concentration of at least 25 mM to maintain equilibrium, unless there is a good reason to use less. Most methods employing the use of an ion-pairing reagent use concentrations less than 10 mM–even as low as 1 mM. We have found that the use of low concentrations of these reagents results in irreproducible retention times. In this method the use of 25 mM myristyltrimethylammonium bromide as ion-pairing reagent stabilised the retention times of the peaks, once the column was fully equili-

Table 1
Precision data for ascorbic acid and uric acid in plasma

Average concentration (μM)	Number of samples in batch	Intra-batch C.V. (%) [a]
Ascorbic acid		
58.1	6	1.3
56.9	6	1.3
58.0	12	0.6
57.6	12	2.0
56.6	7	1.4
Uric acid		
306	6	1.2
281	6	0.8
295	12	0.8
307	12	0.5
288	7	2.0

[a] The C.V.s for ascorbic acid and uric acid determinations over a 2-week period were 1.0% and 3.4%, respectively.

brated with the mobile phase, which may take up to 4–5 days of pumping at 0.5 ml/min. If the column is not fully equilibrated there is a large variation of the retention time of the various peaks between different samples which renders peak identification difficult.

Ideally the sample to be separated should be applied in the mobile phase to avoid upsetting the ion-pair equilibrium but this was not practical in our method. Therefore only 20 μl of sample was injected onto the column; this was small enough not to affect the equilibrium of the column.

The life-time of the analytical column was extended by changing the guard column packing after every 200 samples. The use of a strong buffer at pH 5.5 protected the column from the low pH of the sample. Myristyltrimethylammonium bromide is both an antibacterial agent and a surfactant, which may explain why it was possible to run as many as 1200 samples on one column.

In order to optimise the UV detection, samples were dissolved in the mobile phase and scanned in a UV spectrophotometer. It was found that 262 nm, which is the absorbance maximum for ascorbic acid, was suitable for both uric acid and ascorbic acid. This was chosen because ascorbic acid is present at a lower concentration than uric acid in plasma and also gives a weaker absorbance signal than uric acid which has its absorbance maximum at 285 nm.

The use of 25 m*M* ion-pairing reagent, which improves the equilibrium of the column, may be a major factor in contributing to the reproducibility of this method.

Homocysteine was added to the mobile phase to stabilise the ascorbic acid in its reduced form [8] and EDTA was also added to chelate any transition metal ions which may be present, thus preventing the oxidation of ascorbic acid [9]. To stabilise the ascorbic acid in the reduced form at low pH during storage many methods use metaphosphoric acid, perchloric acid or trichloroacetic acid; these acids also precipitate the plasma proteins [4,10]. These proteins are removed by centrifugation, since they may bind to the packing of the analytical column, shortening the life of the column. Samples which are not immediately analysed for ascorbic acid must be stored frozen at −80°C to ensure the stability of the ascorbic acid. Previous studies have shown that ascorbic acid is stable for a longer period at lower temperature [10]. The present method employed metaphosphoric acid but samples stabilised in perchloric acid could just as easily be run. However, they did not give as clear a supernatant after centrifugation as samples prepared in metaphosphoric acid.

This method has advantages over other procedures in that it is uncomplicated, inexpensive, requires only a simple reversed-phase C_{18} column and UV detector and gives highly reproducible results.

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