ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
14 (1996) 713–719

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Determination of citrate in plasma protein solutions by UV–visible spectrophotometry and ion chromatography

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Received for review 4 July 1995; revised manuscript received 16 October 1995

Abstract

Two procedures were compared for the determination of citrate in plasma protein solution (PPS). Ion chromatography with gradient elution was preferred to an enzymatic–spectrophotometric method for citrate concentrations in the range 0.4–11 mg l^{-1}. Better comparisons of results by both methods were obtained for citrate concentrations at the g l^{-1} level, as dilution of the PPS reduced interferences in the enzymatic procedure.

Keywords: Citrate; Ion chromatography; Spectrophotometry; Plasma protein solution

1. Introduction

Plasma protein solution (PPS) is used in blood transfusions and as a nutrient source for individuals who cannot take solid foods. It is primarily an albumin product, which has been prepared from human blood, but has had the red blood cells and clotting factors removed. As a consequence of the treatment process, PPS can contain citrate and aluminium. Hence, there is interest in evaluating the concentrations of both species in commercial products, particularly as citrate is known to enhance the absorption of aluminium and other metal ions in the body [1–4].

Many of the published methods on citrate concern the analysis of fruit juices [5,6], beverages [7,8] or food [5,8–10], but a few have investigated serum [11–13] or urine [11,12,14]. The most commonly used techniques for the determination of citrate are chromatography [5,7,14–18] and spectrophotometry [9,11,12], but methods involving membrane electrodes [19,20] have also been reported. Singh et al. [14] used isocratic elution with 35 mM sodium carbonate and conductometric detection for the determination of citrate and isocitrate. The response was linear up to 200 μM for citrate and 250 μM for isocitrate, and the signal precision was 9.6% at 25 μM citrate. When 10 μM citrate was added to urine containing

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SSDI 0731-7085(95)01671-6

about 2 mM isocitrate, the recovery was 96.8%. Other chromatographic methods [5,7,15–18] used UV–visible spectrophotometry at a range of wavelengths to detect relevant species. The spectrophotometric methods [9, 11,12] are all based on an enzymatic procedure first reported by Möllering and Gruber [21]. When the method was adapted by Plantá et al. [9] for flow injection analysis, a throughput of 20 samples per hour was achieved.

The enzymatic method has been described [22] as being non-specific, involving long and troublesome procedures which reduced the sensitivity of the determination. The main disadvantage of the enzymatic method is that the citrate lyase has low stability, decreasing in activity by 6% over a 10 min period [9]. Inorganic salts have also been reported to interfere with the enzymatic method, and to overcome this problem more enzyme was added to the solution to be analysed [22]. There is also a dispute as to whether serum solutions should be deproteinized prior to analysis, with some workers stating that proteins do not interfere with the determination [12] and others suggesting that serum citrate values were higher after filtration; in contrast, urine citrate values were not affected.

In this work, the enzymatic spectrophotometric method and a procedure based on ion chromatography, with gradient elution and conductometric detection, were compared for the determination of citrate in plasma protein solutions.

2. Experimental

2.1. Preparation of samples

Protein-free solutions were used for analysis by ion chromatography, whereas the spectrophotometric method used the solutions unfiltered.

Protein-free solutions were prepared using a Centriprep-10 concentrator (Amicon, UK) which contained a membrane filter with a molecular weight cut-off of 10 000.

The filter was prepared by adding 15 ml of 0.9% (m/V) sodium chloride solution (Baxter Health Care, UK) to the container before spinning for 15 min at 2100 rpm at 25°C. The retentate and filtrate were removed and 5 ml of plasma protein solution were placed in the sample container and centrifuged twice for 15 min at 2100 rpm at 25°C. The filtrate was collected and placed in a container after each spin, for determination of citrate. The filter was washed with 3 ml of 0.9% (m/V) sodium chloride solution and centrifuged for 15 min under the above conditions to ensure that no sample was trapped in the membrane. The wash filtrate and any retentate were analysed for citrate, but none was ever detected.

2.2. UV–visible spectrophotometry

A Philips PU8900 UV–visible spectrophotometer was used at 340 nm with a fixed bandpass of 2 nm. The samples were analysed in disposable plastic cuvettes.

The method kit was obtained from Boehringer Mannheim (catalogue number 139 076). Three solutions were provided in the kit, which is normally used to determine citrate in food. Solution 1 contained 1.4 g of lyophilizate, which consisted of glycylglycine buffer (pH 7), malate dehydrogenase (136 U), lactate dehydrogenase (280 U), 6 mg of nicotinamide–adenine dinucleotide (NADH) and stabilizers. Solution 2 contained 50 mg of lyophilizate citrate lyase (12 U) and solution 3 contained a standard citrate solution (0.4 g l^{-1}). In the above, U is a unit of enzyme, which is defined as the amount which will catalyse the transformation of 1 micromole of substrate per minute, or, where more than one bond of each molecule is attached, 1 microequivalent of the group concerned per minute, under defined conditions [23].

The spectrophotometric method for the determination of citrate involves the following reactions:

$$\text{citrate} \xrightarrow{\text{citrate lyase}} \text{oxaloacetate} + \text{acetate} \quad (1)$$

$$\text{oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{L-malate} + \text{NAD}^+ \quad (2)$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{L-lactate} + \text{NAD}^+ \quad (3)$$

where NADH and NAD^+ are nicotinamide adenine dinucleotide in the reduced and oxidized forms, respectively, MDH is malate dehydrogenase and LDH is lactate dehydrogenase. Pyruvate is a decarboxylation product of oxaloacetate. The concentration of citrate is calculated from the amount of NADH which is oxidized to NAD^+, which can be determined by spectrophotometry [13].

The procedure involved the following steps. A 1 ml aliquot of solution 1 was pipetted into a plastic cuvette followed by 1.8 ml of distilled water and 0.2 ml of unfiltered plasma protein solution. The solution was mixed, left to stand for 5 min and then the absorbance (A_1) at 340 nm was measured. The blank solution contained 1 ml of solution 1 and 2 ml distilled water. After A_1 had been measured, 0.02 ml of solution 2 was added to the cuvette and the absorbance (A_2) was recorded after 5 min. the difference in absorbance ($A_1 - A_2$) was calculated for the blank solution and the samples, and the results were converted to citrate concentrations using the equation

$$c = \frac{(A_1 - A_2)v_f m}{abV_s} \quad (4)$$

where c = citric acid concentration (g l^{-1}), V_f = final volume in the cuvette (cm^3), m = molecular mass of citrate (g mol^{-1}), a = the absorption coefficient of NADH at 340 nm (1 mol^{-1} cm^{-1}), b = light path (cm) and V_s = sample volume (cm^3). In this study, the values of V_f and V_s were 3.02 and 0.2 cm^3, respectively, m was 192.1 g mol^{-1}, a was 6300 l mol^{-1} cm^{-1} and b was 1 cm.

Table 1
Gradient elution programme for the determination of citrate in ultrafiltered plasma protein solutions by ion chromatography

Time (s) [a]	Eluent 1, 75 mM NaOH (%)	Eluent 2, distilled water (%)	Valve [b]
0	4	96	Closed
0.3	4	96	Open
8.3	50	50	Open
13.3	50	50	Open
14.3	4	96	Open

[a] The composition of the eluent changed linearly between the times stated.
[b] Valve is kept shut to allow the eluents to stabilize. After the programme has been running for 0.3 s, the valve is automatically opened and the sample is injected.

2.3. Ion chromatography

A Dionex 4000i chromatograph was used with a conductivity detector, a gradient pump and an eluent degas module. The instrument was

Table 2
Comparison of citrate concentration (mg l^{-1}) in plasma protein solutions

Sources	Ion chromatography[a]	UV–visible spectrophotometry	
		Operator 1	Operator 2
4.5% Albumin solution, Immuno Ltd.	0.4 ± 0.2	3.8	1.00
SNBTS[b] (batch 3304–82200)	3.1 ± 0.2	15.4	6.9
5% Albumin solution, Armour Pharmaceuticals	11.0 ± 0.2	9.6	7.7
SNBTS[b] (batch 3306-82370)	1.3 ± 0.2	9.6	1.5
4.5% Albumin solution, Elstree Laboratories	0.7 ± 0.1	11.5	1.3
Freeze-dried plasma protein (5 ml of water required for reconstitution) which had been partially filtered during production, SNBTS[b]	3.85 ± 0.04[c]	3.9[c]	3.7[c]
Freeze-dried plasma protein (2 ml of water required for reconstitution) which had not been filtered during production, SNBTS[b]	1.12 ± 0.01[c]	0.9[c]	0.9[c]

[a] The ± values are errors derived from the calibration error.
[b] Scottish National Blood Transfusion Service (SNBTS), Edinburgh.
[c] Concentration in g l^{-1}.

equipped with an AS5 column and AG5 guard column. An anion micromembrane suppressor (AMMS) was used to reduce the background conductivity of the eluent and improve the detection limit of citrate.

Two eluents were used. Eluent 1 was 3 g l^{-1} (75 mM) sodium hydroxide solution and eluent 2 was distilled water. The flow rate of the eluents was 2 ml min^{-1}. The gradient elution programme optimized for the determination of citrate in the filtrate of the plasma protein solutions is given in Table 1. Between the times stated in the table, the gradient changed linearly to the next eluent composition. A detector range of 30 μS was used for the analysis of 50 μl of ultrafiltered plasma protein solutions. Fig. 1 shows examples of the chromatograms recorded for 15 mg l^{-1} citrate standard and a plasma protein solution. Using the programme given in Table 1, the citrate peak was produced after about 13.5 min. Each analysis took about 30 min, as time was required to re-equilibrate the system with the initial eluent composition.

The AMMS was regenerated continuously with 1.96 g l^{-1} sulphuric acid solution flowing under a pressure of 12 psi.

2.4. Citrate calibration solutions

A 1000 mg l^{-1} citrate stock standard solution was prepared by dissolving 1.531 g of trisodium citrate dihydrate (Aristar grade, Merck, UK) in 1 l of distilled water. A working standard solution of 100 mg l^{-1} citrate was prepared by dilution of the stock standard solution with distilled water. The calibration solutions were prepared to contain 0, 3, 5, 10 and 15 mg l^{-1} citrate.

2.5. Plasma protein solutions

The plasma protein solutions were obtained from a variety of manufacturers, as shown in Table 2. The solutions contained either 4.5, 5 or 20% albumin. Two of the samples had been freeze-dried by the supplier (Scottish National Blood Transfusion Service) to lengthen the shelf-life of the samples. The freeze-dried samples were reconstituted with distilled water according to the manufacturer's instructions. The plasma protein solutions also contained sulphate, phosphate, citrate, sodium and chloride.

Table 3
Recovery of citrate added to plasma protein solutions as determined by ion chromatography

Sample	Before citrate addition (mg l^{-1})	After citrate addition (mg l^{-1})	Recovery [a] (%)
Citrate solution	—	12.2 ± 0.2	—
SNBTS	3.1 ± 0.2	15.8 ± 0.2	104.1
4.5% Human albumin solution, Immuno Ltd.	0.4 ± 0.2	11.7 ± 0.2	92.6

[a] Recoveries were calculated by the equation

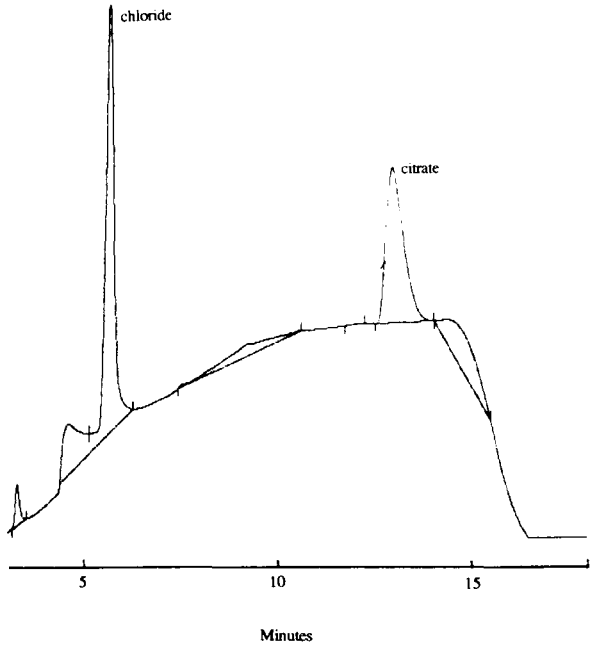
$$\text{recovery (\%)} = \frac{[\text{after}] - [\text{before}]}{[\text{citrate standard}]} \times 100$$

where [ ] represents concentration.

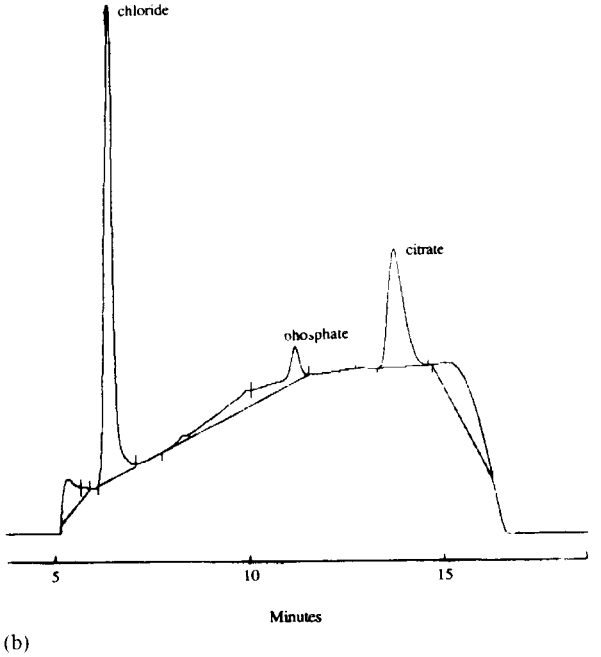
3. Results

3.1. Spectrophotometry

The manufacturers of the enzymatic kit recommend that samples containing >0.4 g l^{-1} citrate should be diluted before analysis, but that the concentration should be at least 20 mg l^{-1}. As the plasma protein solution under study in this work had a range of citrate concentrations, it was expected that some samples would be analysed undiluted and others after 10-fold dilution with distilled water. Initial studies with various plasma protein solutions indicated that there was very little change in absorbance after the prescribed time of 10 min, irrespective of whether the samples were diluted or not. The results in Fig. 2 show the change in absorbance over a period of 50 min for the 0.4 g l^{-1} citrate standard supplied with the kit, and for two plasma protein solutions, one of which was analysed undiluted and the other after 10-fold dilution. The citrate standard shows the expected change in absorbance after 10 min, as does the diluted human albumin solution from the Scot-



(a)



(b)

Fig. 1. Ion chromatograms of (a) 15 mg l^{-1} citrate standard and (b) filtrate of a plasma protein solution, obtained with gradient elution using sodium hydroxide–water eluent.

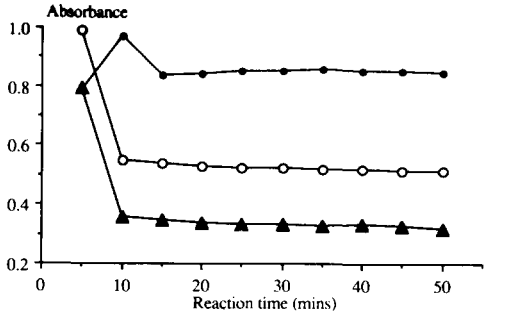


Fig. 2. Change in absorbance of NADH at 340 nm with time for the determination of citrate using an enzyme-based reaction. (○) Reference citrate standard solution (0.4 g l^{-1}); (●) stable plasma protein solution, Scottish National Blood Transfusion Sevice (SNBTS), Edinburgh; (▲) human albumin solution, SNBTS (diluted 10-fold).

tish National Blood Transfusion Service (citrate concentration about 1.12 ± 0.01 g l^{-1}). In contrast, there is only a slight change in the absorbance over the period of the experiment for the second plasma protein solution, undiluted 20% human albumin solution from Elstree Blood Products (citrate concentration about 1.2 mg l^{-1}).

Further tests indicated that the change in absorbance with time differed for various samples and was not repeatable, especially for undiluted plasma protein solutions with a lower citrate concentration, which were analysed undiluted.

A set of samples was sent to two analysis who used the enzymatic kit at Glasgow Royal Infirmary and the Scottish National Blood Transfusion Service, Edinburgh. The results obtained are given in Table 2. Poor agreement was achieved for the samples with lower concentrations of citrate (mg l^{-1} level). However, better agreement was achieved for the samples containing about 3.9 and 0.9 g l^{-1} citrate.

3.2. Ion chromatography

When the gradient elution programme given in Table 1 was used, the citrate peak was detected on a reasonably stable baseline when the eluent composition was 50:50 sodium hydroxide–water. A chloride peak was observed in all chromatograms (e.g. Fig. 1), although the source is likely to have been different for the calibration solutions and the

samples. For the plasma protein solutions, the chloride contamination was probably caused by residual levels of sodium chloride remaining on the filter after conditioning. As the calibration solutions were not ultra-filtered, the chloride peak was more likely to have been caused by impurities in the citric acid or the containers used for preparation of solutions. Irrespective of the source of chloride, no interference was caused on the measurement of the citrate peak, which was also well resolved from the phosphate peak in the chromatograms of the plasma protein samples.

Citrate at a level of about 12 mg l^{-1} was added to two solutions of ultrafiltered plasma protein and the recovery levels where calculated. The results in Table 3 indicate that close to a quantitative signal recovery was achieved. When the samples analysed by the spectrophotometric method were also analysed by ion chromatography, the results in Table 2 were obtained. At the higher concentration level, the results between the techniques are comparable, but agreement is poor for most of the samples containing mg l^{-1} concentrations of citrate. The spectrophotometric results obtained by operator 2 are more similar to those by ion chromatography, although the agreement is far from satisfactory.

The within-run precision, based on $\pm$ one standard deviation, was found to be 1% at 11.8 mg l^{-1} citrate and 9% at 2.1 mg l^{-1} citrate. When two plasma protein solutions were analysed on three separate days, the citrate concentrations obtained were 2.1, 3.0, 2.2 mg l^{-1} and 0.3, 0.4, 0.3 mg l^{-1}, indicating acceptable between-run repeatability. The detection limit of citrate in aqueous solution was calculated (based on 3σ) to be 40 μg l^{-1}. The detection limit for PPS, taking the sample preparation procedure into account, was 0.2 mg l^{-1}. The conductivity response curve was linear to at least 15 mg l^{-1} (the maximum considered here), although different μS ranges were required on the ion chromatograph.

4. Conclusion

The study has shown that the UV–visible spectrophotometric method is not suitable for the determination of citrate in PPS at concentrations typical of many samples i.e. 0.4–11 mg l^{-1}. In contrast, the ion chromatographic method seems to give satisfactory results and is preferred. Although it was necessary in this work to ultrafilter the PPS prior to analysis by ion chromatography. Dionex now offer columns which are claimed to be able to cope with solutions containing up to 90% protein.

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