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Analysis of citrate in low-volume seminal fluid samples using a time-gated measurement of europium luminescence

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ABSTRACT

This work aims to develop and validate a rapid analytical method that enables the measurement of citrate in seminal fluid samples. Samples were obtained from men who were within 9 weeks of a vasectomy operation. Two age ranges were examined, between 40–43 and 50–53 years old, with nearly 100 samples in each case. No patient clinical history was available for this anonymous study, simulating a random screening cohort. The concentration of citrate in 0.5 μL seminal fluid samples was assessed, using a europium emission luminescence method. This involves the ratiometric analysis of two well-separated europium(III) emission bands. Spectral data were obtained using a time-gated spectrometer whose construction and modification is described. Citrate values were confirmed by independent measurements using a citrate lyase enzymatic assay and by 700 MHz ^{1}H NMR analysis of the seminal fluid. Citrate concentrations were not statistically different between age groups and averaged 35.0(±14.6) mM for the 40–43 group, and 28.2(±12.7) mM for the 50–53 cohort; in each case a polymodal distribution was observed.

1. Introduction

Citrate is a key metabolite in mammalian systems. It is normally found in relatively low concentration, as it is transformed by aconitase enzymes in the tricarboxylic acid cycle. Thus, in human serum citrate levels are in the range of 0.06–0.14 mM [1]. There is one major exception to this situation. In healthy males, citrate levels in prostatic tissues and fluids are much higher averaging 94(±32) mM in expressed prostatic fluid [2]. These high levels of citrate are also found in seminal fluid samples, where a healthy person is reported to maintain a level of 25.4(±3.4) [3] to 28.9 mM [4]. This follows from the fact that seminal fluid is normally composed typically of 30 to 40% of prostatic fluid. The elevation of citrate levels in these fluids has been shown to be due to the suppression of the activity of the mitochondrial-aconitase enzyme, induced by elevated zinc levels. This enzyme normally serves to catalyse isomerisation of citrate to isocitrate in the Krebs cycle [5,6].

In prostate cancer, citrate values in prostatic fluids are reduced significantly [2,5–7] as levels of zinc within epithelial cells fall, due to mis-regulation of a zinc transporter protein. This metabolic change reactivates the Krebs cycle, so that citrate levels fall; reductions of up to a factor of ten have been reported to occur, coupled to a lowering in the concentration of zinc, magnesium and calcium [8,9]. Intriguingly, these metabolic variations in citrate levels have been hypothesised to precede the histopathological changes that are revealed by biopsy of prostate tissue, leading to the hypothesis that the citrate level in expressed prostatic fluid (and seminal fluid by extrapolation), is a viable early marker for prostate cancer [6]. Alternate biomarkers for early diagnosis of prostate cancer are actively being sought as the prostate specific antigen (PSA) test has been shown not to have sufficient sensitivity and specificity to permit accurate diagnosis or monitoring [10].

Support for the examination of citrate as a metabolic marker for prostate cancer has come from a series of magnetic resonance spectroscopy and imaging studies, examining citrate levels in patients with cancer, benign prostate hyperplasia (adenoma) versus healthy controls [11–15]. Reductions in citrate concentration (often calibrated as [choline + creatine] to citrate ratios) of a factor of between 3 and 5 were found for high grade disease, and proportionate changes have been reported in the analysis of seminal fluid samples [16].

Levels of citrate in seminal fluids may be depleted in certain other clinical conditions. Citrate levels in seminal fluid are also reduced in infertile men [3]. Reductions of 25% in normozoospermia and 34% in oligozoospermia have been reported, with parallel reductions in zinc and magnesium levels. Patients suffering from benign prostate hyperplasia (adenoma) do not normally exhibit

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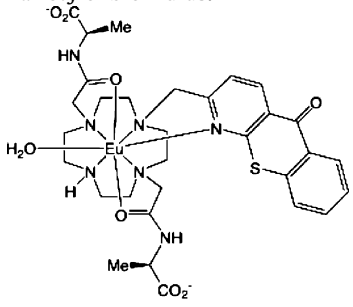
reduced citrate levels [16]. In prostatitis patients, inflammation of the prostate may lead to reductions in citrate in expressed prostatic fluid by up to 200–300%, due to decreased secretion [17]. Prostatitis affects about 7% of the adult male population and a reduction in citrate levels of up to a factor of 2.5 has been reported for patients with the most common form, abacterial prostatitis (sometimes termed prostatodynia) [18]. Therefore, if citrate levels in prostatic or seminal fluid are to be used to signal the onset or presence of prostate adenocarcinoma, caution must be exercised in assessing the possibility of these other conditions—each of which normally presents symptoms earlier. In this respect, readings taken as a function of time (i.e. "active monitoring") may be preferred, as the development of prostate cancer may be hypothesised to lead to a continuing reduction in citrate levels, whilst prostatitis may be indicated by the attainment of a limiting value.

It should be noted that it is generally appreciated that care must be taken to minimise the risk of bacterial contamination of the sample to be analysed, as many common bacteria (e.g. enterobacteria, lactobacilli and streptococci) can use citrate as a 'fuel' in their proliferation [19,20].

Classically, levels of citrate in bio-fluid samples are estimated using enzyme-based assays, often using citrate lyase [1]. These methods usually require sample pre-treatment steps and can be quite time consuming. An alternative, much faster method has been developed and reported in a preliminary communication [21]. It is based on the ratiometric analysis of europium luminescence of the complex $[Eu.L^1]^+$ that binds citrate reversibly and selectively in aqueous media. Similar luminescence methods have been used to establish pH [22], lactate [21] or urate [23] concentrations in a variety of bio-fluids.



$[Eu.L^1]^+$

In this work, we have set out to develop and validate a convenient and rapid analytical method that allows the measurement of citrate levels in seminal fluid samples. Samples were obtained from men who had recently undergone a vasectomy and hence were assumed not to have been infertile, i.e. were neither normozoospermic, nor oligozoospermic. Two age ranges were selected, between 40–43 and 50–53 years old, with nearly 100 samples examined in each case. No clinical history was available for this anonymous study, so that healthy men were likely to be examined as well as those with other conditions such as non-bacterial prostatitis, benign prostate hyperplasia or prostate cancer.

2. Materials and methods

2.1. Reagents

The Eu complex $[Eu.L^1]Cl$ was prepared as described in the preliminary communication [21]. Salts and HEPES buffers used were of AnalaR^{TM} grade (VWR-Jencons, UK) and solutions were made up using water that has a conductivity of less than 0.04 $\mu S\,cm^{-1}$, obtained from the 'PuriteSTILLPlus' purification system (Purite Ltd., Thame, UK).

2.2. Instrumentation

An in house-built time-gated spectrophotometer was used to measure and record lanthanide emission spectra (www.fscanltd.com). This was designed to maximise Eu(III) emission detection sensitivity and reduce physical size. A pulsed LED excitation source (Nichia: 365 nm, 50 μs, 200 Hz, 250 mW) was placed in a purpose built sample chamber, designed to accommodate commercially available cuvettes (e.g. 12 mm × 12 mm or 50 μL to 3.5 mL). Luminescence from the sample was collected at 90° to the excitation source and focussed onto the entrance slit of a monochromator (Acton), adjusted to provide a 2 nm band-pass. The emission was detected using a photon counting photomultiplier module (Hamamatsu) and the signal acquired using a PC-based National Instruments data acquisition card. Eu(III) emission spectra were obtained in the range 550–720 nm in 1 nm increments using low volume, plastic cuvettes (50 μL, Eppendorf UVette®).containing the emissive Eu complex (20 μM). A 10 μs time-delay was applied prior to a 1 ms time-gate for the spectral acquisition, in order to eliminate scattered light, avert auto-fluorescence and avoid any residual short-lived fluorescence. All components were housed within a machined polymethylmethacrylate shell, with only the sample holder and PC input devices externally accessible, as the instrument was controlled using a modified PC, running a Windows platform. This provides the timing sequence for the LED and photon-counting detector, and controls the monochromator, as well as providing power to every instrument component.

Instrument calibration was carried out in two respects. The wavelength of the monochromator was calibrated (±0.2 nm), using a standard mercury-argon lamp. The spectral response of the spectrometer was then determined using a calibrated tungsten lamp and a correction function generated. This function was applied to each spectrum as it was acquired. Both raw and corrected spectra were saved within the recorded data file. The analyte concentration calculations were only made using the corrected spectrum. The calibration process, instrument accuracy and response profile (dark-count, validation and standard sample measurement) were checked every month. Over a period of 12 months, no drift or change in accuracy was observed for each standard sample value recorded, within a 3% measurement error.

The instrument control software was written in LabView 7.1 (National Instruments) and was programmed to allow measurement of multiple scan sequences allowing the correlation of the specified spectral ratio with an embedded calibration curve. It permits the direct display of the analyte concentration, once the chosen triplicate scan sequence (<3 min) has been completed and averaged. The software was adapted for use with either simulated seminal or prostatic fluid backgrounds, consisting of 0.1 M sodium chloride, 0.04 M potassium chloride, 5 mM magnesium chloride, 4 mM calcium chloride, 2 mM zinc chloride, 3 mM sodium bicarbonate, 10 mM sodium lactate and 0.3 mM human serum albumin (HSA). Samples were diluted 100 fold using a buffer solution containing 0.1 M NaCl, 0.1 M HEPES and 0.1 M sodium lactate at pH 6.55. The calibration curve was recorded in triplicate with standardised stock solutions of AnalaR^{TM} grade samples of disodium citrate, using a 15-point calibration protocol. Calibration curves were averaged and stored. The calibration protocol was checked on a weekly basis using a set of pre-made standard samples whose recorded values did not vary, within the error of the measurement, over a period of one year. Citrate level calculations were made using selected Eu emission intensity ratios at 605–625 nm vs. 575–605 nm. Spectra were scanned in 1 nm increments, and each spectrum recorded was corrected using a point-by-point subtraction of the average dark count calculated from the region 555–565 nm. In order to minimise light exposure and scan time each spectrum was recorded

three times between 555 and 630 mm, and the averaged spectrum stored.

2.3. Sample analysis by luminescence emission measurement

Seminal fluid samples were received from a local analytical laboratory, where spermatozoa counts are assessed optically to assess vasectomy outcome. Ejaculate samples had been provided voluntarily, to permit fertility assessment, from patients who had not ejaculated for >48 h. In every case, samples were obtained two months after the vasectomy operation.

The samples were selected on the basis of age, defining two groups between 40–43 and 50–53 years old. Samples were collected in batches of 50 and were stored at 4 °C; triplicate citrate analyses were carried out on each sample the day after receipt and samples were subsequently discarded in accordance with the Human Tissue Act guidelines. All measurements were carried out in a category 2 level clean laboratory facility, authorised for use by the Chemistry Department, Durham University. Local ethical approval was obtained to carry out this study. Each sample was kept in sterile containers at room temperature at the commercial laboratory and subsequently was held in a sterile freezer (−20 °C) in the original re-sealed container.

Sample measurement used a 50 μL Eppendorf UVette® cuvette (Fisher Scientific Ltd., UK) and when the Eu(III) complex was premixed with the buffered dilution solution, a similar speed and measurement accuracy was achieved. Samples of seminal fluid (0.5 μL) were diluted 100 fold using a buffer solution containing the Eu(III) complex (20 μM) in 0.1 M HEPES buffer, 0.1 M NaCl, 0.1 M sodium lactate at pH 6.55.

2.4. Data acquisition and analysis

Readings of citrate concentration were taken in triplicate. The instrument software was set to get an average of triplicate scans to establish citrate concentrations, so each sample was measured 9 times to establish the mean citrate concentration. The mean error in analysis was 3%, with a limit of detection of 3 mM, when using a 0.5 μL sample of seminal fluid using a Gilson positive displacement pipette. The overall measurement protocol took under 10 min, using a total of 1.5 μL of the bio-fluid for each sample.

Analysis of the variation of sample frequency with citrate concentration was undertaken using standard statistical packages running in Origin™. The equation used to model a Gaussian distribution (data shown in Fig. 5) was based on the following function:

$$f(x) = \frac{A}{W\sqrt{\pi/2}} \exp\left[\frac{-2(x - X_c)^2}{W^2}\right]$$

where X_c is the mean citrate concentration value (mM) quoted and W is the half-width/half-maximum. For the 50–53 age group, X_c = 32.8 mM (W = 22.5); for the 40–43 age group, X_c = 38.8 mM (W = 25).

2.5. Sensor validation: enzymatic and ^{1}H NMR analyses

For the seminal fluid samples, citrate and lactate concentrations were also assessed using a citrate lyase or lactic acid dehydrogenase enzyme assay kit (Megazyme Ltd., Eire). The measurements were carried out following the manufacturer's protocol, using 10 times diluted and filtered samples (Amicon Ultra 3 kD centrifuge (3000 rpm) filters, Millipore; 15 min). Changes in measured absorbance (Unicam UV2, Plastibrand Macro-PMMA cuvettes, total volume of 3 mL) were monitored until an equilibrium reading was reached (typically taking up to 1 h). Calculations of concentration were made using the appropriate dilution factor. Measurements were made in triplicate and the mean value recorded. The enzyme detection method required a minimum sample volume of 0.1 mL. Sample analysis took on average 3 h; the measurement required a sample pre-treatment protocol involving filtration to eliminate interfering species, such as protein and fatty acids. Measurements were made in triplicate and the mean value recorded.

For 12 ejaculate samples, citrate values were also determined at 16.5 T by ^{1}H NMR spectroscopy, using a Varian VNMRS-700 instrument. One-dimensional spectra (45° pulse, 128 transients, 11,261 Hz spectral width, 32k time domain data points, 1 s relaxation delay, 2.91 s acquisition time) were recorded at 295 K using a standard solvent suppression pulse sequence. Spectra were processed with an 0.3 Hz line-broadening function. Samples were subjected to centrifugal filtration (3000 rpm, 15 min) and a seminal fluid sample (0.25 mL) was diluted 3-fold using deionised Purite™ water (C = 0.18 μS cm^{-1}). The sample was lyophilised and solvent exchanged (3 times) using deuterium oxide prior to NMR analysis in D_2O. Chemical shifts are quoted relative to the HDO peak at δ 4.72, and spectral assignments agreed with literature data reported for seminal fluid analyses [24,25]. Incremental addition of a standard disodium citrate solution in D_2O (Aldrich, ACS 99.5%) to a seminal fluid sample containing no citrate, allowed calibration of the NMR citrate concentration measurement. The mean values measured for each of the three methods used were consistent, within 10%. The limit of detection of citrate in this NMR analysis was estimated to be 0.5 mM, based on an instrument S/N ratio of 500:1, using a 4:1 S/N ratio threshold to define the limit.

In some of the first seminal fluid samples to be measured, pathogenic bacterial contamination was encountered. Samples from volunteers had been obtained under "uncontrolled conditions": the only guidance issued was to avoid the prior use of spermicidal lubricant. Samples were held at room temperature at the commercial laboratory and only after transfer to Durham University were they kept at <4 °C. Contamination may be due to the presence of different bacterial species, such as enterobacteria, which are known to reduce citrate levels quickly via anaerobic metabolism [19,20]. Every sample with a citrate value of lower than 20 mM was subsequently incubated at 310 K for 48 h and re-measured. If bacterial contamination was present, the citrate value fell to zero, and this sample was discarded. Control experiments, re-adding known amounts of citrate (up to 20 mM) confirmed this time and temperature dependent reduction. No change in citrate values was found over a period of 2 months for 10 control samples stored below 4 °C. For every sample, repeat measurements were taken after standing for 7 days at 25 °C; if no change in the measured value was noted, the sample was deemed free of bacterial contamination.

As a separate control experiment, a common bacterial growth medium, tryptose phosphate broth (Sigma–Aldrich Ltd., UK), was used with the seminal fluid samples of suspected bacterial contamination in 10-fold dilution. The enriched growth medium was incubated at 310 K for 48 h. This allowed visual confirmation of extensive bacterial growth for samples with bacterial contamination. These samples were not used in the data analysis. Subsequent samples were stored in vials containing Virkon™ to give a final solution that is 0.2% in the anti-bacterial agent. Readings of citrate concentration did not change on storage over a period of 3 months for every sample treated and tested in this manner not even if a small amount of bacteria was deliberately introduced to the samples within 5 days of sample obtainment, The Virkon solution is estimated to have a shelf-life of up to 7 days. The presence of this anti-bacterial agent (in an 0.1–1.0% concentration range) did not change the measured values obtained by the Eu emission assay.

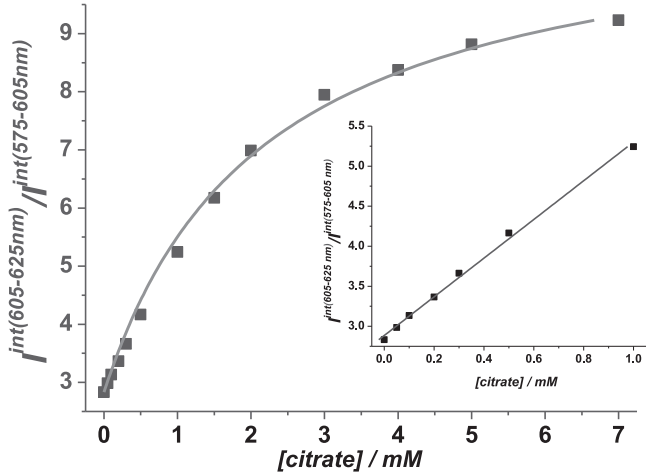


Fig. 1. Calibration curve showing integrated emission intensity in terms of the ratio of the $\Delta J = 2$ bands (605–625 nm) to the $\Delta J = 1 + \Delta J = 0$ transitions (575–605 nm), caused by the modulation of Eu(III) luminescence for $[Eu.L^1]^+$ associated with reversible binding of citrate to the metal centre (pH 6.5, 0.1 M HEPES, 0.1 sodium lactate, 0.1 M NaCl, 20 μM complex). The line shows the fit to the experimental data points using an iterative non-linear least squares procedure. The insert highlights the linear range ($R^2 = 0.996$).

3. Results and discussion

The synthesis, characterisation and anion selectivity profile of the europium complex $[Eu.L^1]Cl$, used in this study, has been reported in a preliminary communication [21]. This preliminary work had established the feasibility of using this complex to determine citrate concentrations in expressed prostatic fluid. In the preliminary work, the intensity ratio of the emission bands centred at 616 and 593 nm was used to report on the variation of citrate concentration in a solution sample.

Here, we set out to determine citrate in seminal fluid samples and develop an analytical protocol for measurement. The diluent used was a standard (100 mM) HEPES saline buffer, maintaining pH at 6.55, to which sodium lactate had been added to give a solution that has a lactate concentration of 100 mM. In preliminary work, it had been established that lactate is the primary interferent for these analyses [21] and is typically present in such samples within the concentration range 5–60 mM. The apparent selectivity of $[Eu.L_1]^+$ for citrate over lactate is 89:1 (log *K* cit 4.41 lac 2.47) (298 K, 0.1 M NaCl, 0.9 mM sodium phosphate, 4 mM KCl) as assessed by the ratio of apparent equilibrium constants for 1:1 complex formation under the stated conditions. Thus, interference from variable sample lactate concentrations was minimised. The use of the 10 μs time-gate in spectral acquisition avoids observation of unwanted fluorescence that can lead to a sloping baseline in the europium emission spectral region, jeopardising accurate ratiometric detection.

Samples of seminal fluid (0.5 μL) were diluted one hundred fold. This allowed measurements of the resultant 50 μL solutions to be taken in the linear range of the calibration curve (Fig. 1). The calibration curve was generated using solutions of pre-determined molarity, made up using a simulated seminal fluid 'blank' containing 0.1 M NaCl, 0.04 M KCl, 5 mM $MgCl_2$, 4 mM $CaCl_2$, 2 mM $ZnCl_2$, 3 mM $NaHCO_3$, 10 mM NaLactate and 0.3 mM HSA at pH 6.55. This citrate-free artificial seminal fluid sample was then diluted 100 fold using pH 6.55 buffer solution containing HEPES, NaCl and sodium lactate of 0.1 M molarity. The calibration curve was generated following incremental addition of citrate stock solution using trisodium-citrate salt of established purity. The linear range extends from 0.03 to 0.75 mM citrate, corresponding to citrate levels in the undiluted seminal fluid sample of 3–75 mM, with a limit of detection of 0.03 mM, corresponding to 3 mM in the original seminal fluid sample.

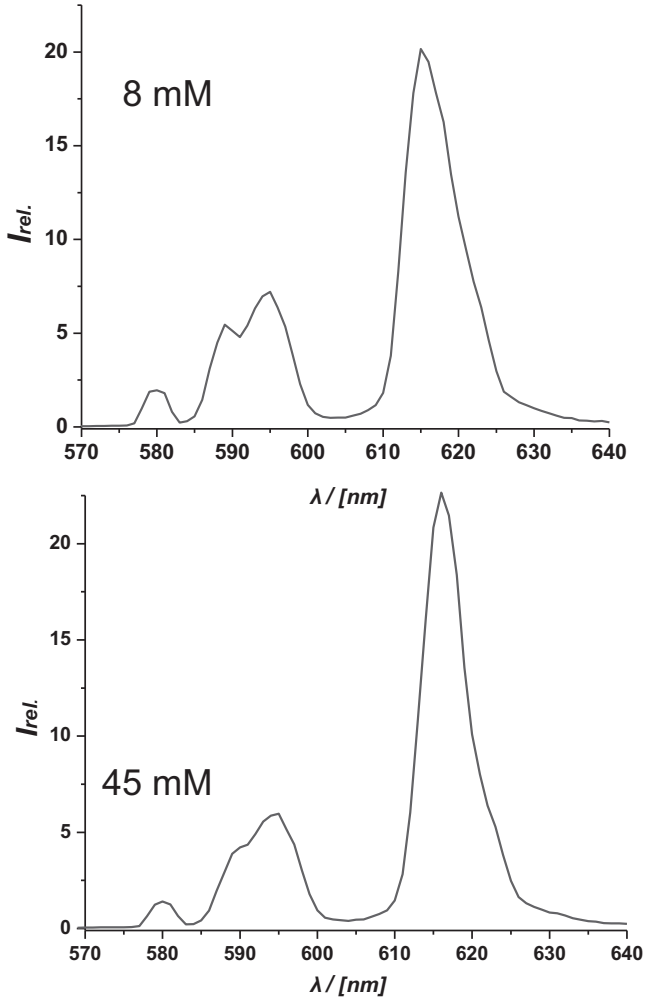


Fig. 2. Representative emission spectra Eu(III) recorded for diluted seminal fluid samples containing 8 and 45 mM citrate, using the time-gated spectrometer (10 μs time-gate, 1 ms time-gate for acquisition, 20 μM complex in 50 μL total volume, 295 K, pH 6.55, 0.1 M HEPES, 0.1 M sodium lactate, 0.1 M NaCl). The ratios of the integrated areas of the $\Delta J = 2$ manifold (605–625 nm) vs. the $\Delta J = 1$ and $\Delta J = 0$ transitions (575–605 nm) are 3.05 (upper, 8 mM) and 4.35 (45 mM).

Representative Eu(III) emission spectral profiles for samples containing 0.08 and 0.45 mM citrate (i.e. diluted one hundred fold from 8 mM to 45 mM) reveal the significant change in that *ratio* of the intensity of the selected emission bands in the range, associated with selective and reversible ligation of citrate to the Eu(III) centre [26] (Fig. 2). Binding of the citrate anion to the Eu centre occurs via formation of a 5-ring chelate, involving cooperative ligation of the hydroxyl and α-carboxylate groups [26]. In the citrate ternary adduct, the Eu emission spectrum is distinctively different from corresponding adducts with anions such as carbonate, lactate and phosphate. In particular, the intensity of the hypersensitive $\Delta J = 2$ transition (range 605–625 nm) is increased relative to the $\Delta J = 0$ (580 nm) and the magnetic-dipole allowed transitions of the $\Delta J = 1$ bands at 585–600 nm. The $\Delta J = 2$ to $(\Delta J = 1 + \Delta J = 0)$ intensity ratio thus reports the equilibrium concentration of citrate that binds to the metal centre.

In order to assess the accuracy of the Eu-based measurements of citrate in seminal fluid, fifty samples were also analysed using an enzymatic assay, based on the activity of citrate lyase. Measurements of citrate made using the luminescence and enzymatic assays (Fig. 3) correlated very well ($R^2 = 0.985$, slope = 0.97), consis-

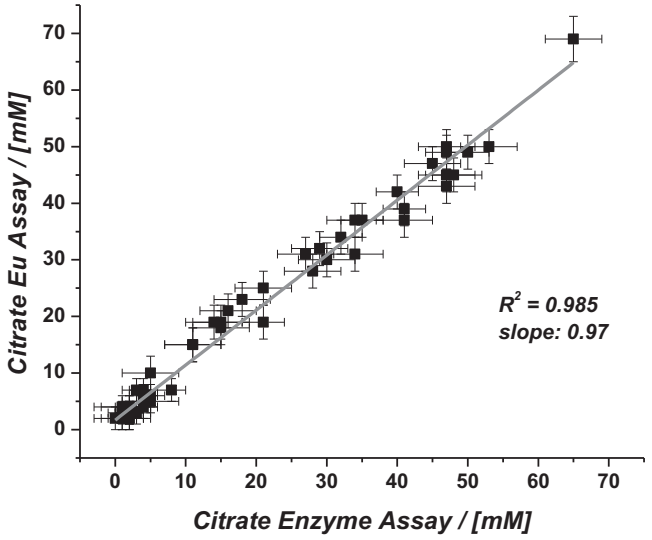


Fig. 3. Correlation of citrate concentration values determined using the Eu(III) luminescence assay vs. an enzymatic assay based on citrate lyase activity.

tent with the validity of the luminescence method. The enzymatic series of triplicate measurements for a given seminal fluid sample took 3 h in total to complete, compared to less than 10 min for the triplicate luminescence assay.

Independent verification of these results was undertaken by analysing the ^{1}H NMR spectrum of a representative set of twelve seminal fluid samples. The seminal fluid sample was diluted by a factor of three into water, lyophilised and re-dissolved in D_2O; this process was repeated prior to spectral analysis at 700 MHz. The distinctive ^{1}H NMR resonance of the two diastereotopic methylene groups (four protons, appearing as an AA′ quartet) in citrate was apparent, resonating close to the AA′B spin system (two protons) of the $CH_2CO_2^-$ protons in the metabolite aspartate (Fig. 4). These assignments were verified by adding authentic aspartate of established purity to the NMR sample and were in agreement with literature data [24,25] (Table 1).

Calibration of the citrate concentration in this NMR method was achieved by incremental addition of a standard solution of sodium citrate in D_2O of known molarity. Values determined by

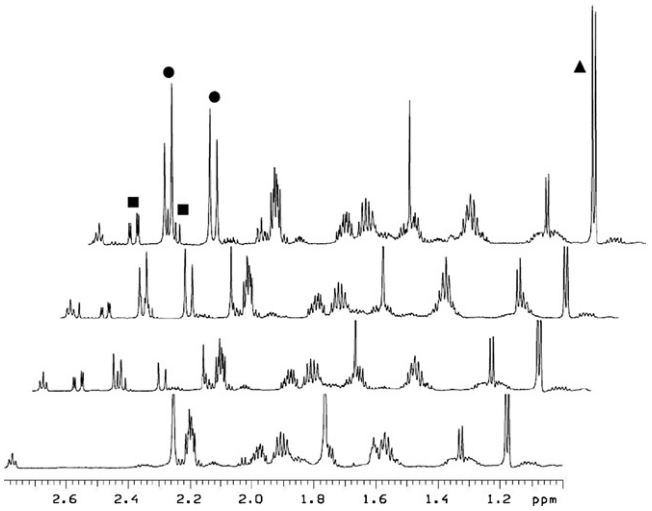


Fig. 4. ^{1}H NMR spectra of seminal fluid samples containing (top to bottom) citrate levels of 58, 29, 13 and <0.5 mM (700 MHz, D_2O, 295 K). Resonances due to citrate (4H, dd, CH_2, *circle*), aspartate (2H, ddd, CH_2, *square*) and lactate (3H, d, CH_3, *triangle*) resonances are indicated, [d = doublet].

Table 1
Concentrations of citrate[a], aspartate and lactate[b] in representative seminal fluid samples, measured by ^{1}H NMR integration (700 MHz, D_2O, 295 K).

Sample number	Citrate (mM)	Aspartate (mM)	Lactate (mM)
20	78(75)	28	53(51)
35[c]	<0.5(<3)	<0.5	45(44)
37	58(64)	34	48(42)
44	13(15)	26	34(34)
46	29(32)	30	29(27)
72	9(8)	44	58(54)
74	11(10)	40	33(34)
75	7(8)	22	28(31)
80	12(14)	22	28(26)
92	5(4)	20	35(35)
100	10(11)	30	16(21)

[a] Values in parenthesis refer to determinations using the Eu assay method.
[b] Values in parenthesis refer to lactate determined using a commercial enzyme kit, based on lactic acid dehydrogenase.
[c] This sample was contaminated by bacteria.

the NMR method agreed with those measured by the luminescence/enzymatic methods, within an error of 10%. In parallel, the concentrations of aspartate and lactate in these samples were determined. Aspartate is actively transported into prostate epithelial cells and serves as the source of the oxaloacetate that is used in citrate synthesis [27]. Levels of aspartate in seminal fluid averaged 29.6 mM. Significant concentrations of lactate were also measured and averaged 38 mM, but no significant correlation was found between citrate concentrations and either aspartate or lactate, in this sub-set of samples.

3.1. Citrate variation in seminal fluids

Citrate readings for 97 and 96 seminal fluid samples in the age range 40–43 and 50–53, respectively, gave rise to polymodal distributions (Fig. 5). The mean citrate concentrations recorded in the lower and higher age ranges were 35.0 mM with a standard deviation of 14.6 mM and 28.2(SD ± 12.7) mM. However, it is very clear that a polymodal distribution is present in each case, with one distinct cohort present above 15 mM giving rise to a near-Gaussian distribution. A calculated Gaussian distribution is shown (Fig. 5) for the 40–43 age group (84 samples), centred around a mean citrate value of 38.8(±11.6) mM (half-width/half maximum 22.5 mM). A similar procedure for the cohort of 78 samples in the 50–53 age group centred around a mean of 32.8(±9.5) mM (half-width/half-maximum 25 mM) also shows a good correspondence, above the hypothetical <15 mM cut-off point.

These data correlate quite well with the most recent data reported for healthy patients of undefined age 25.4 (±3.4) mM [3], in which putative bacterial prostatitis and cancer patients were excluded from the analysis. No short term study has previously reported citrate levels in recently vasectomised men. However, for men vasectomised 8 years earlier, mean citrate levels (median age 45, range 35–70) were reported to be 26 mM compared to 28.8 mM for an age-control matched non-vasectomised cohort [28]. Thus, a significant change in citrate concentration was not observed as a consequence of the vasectomy.

Six (age range 40–43; p value < 0.045) and 10 (range 50–53, p value < 0.065) percent of citrate values were less than 10 mM in each case. Whilst some of the samples with citrate values in the range 9–15 mM could be attributed to patients with inflammation, as this can reduce citrate secretion by a factor of 2.5 [17,18], the *very* low values are more difficult to reconcile, unless the prostatic fluid flow is blocked, or citrate metabolism has been triggered *in vivo*, as occurs when malignancy is present. Evidently, a more extensive study is warranted for patients of defined clinical status, to gather sufficient data to allow the utility of citrate analysis in sem-

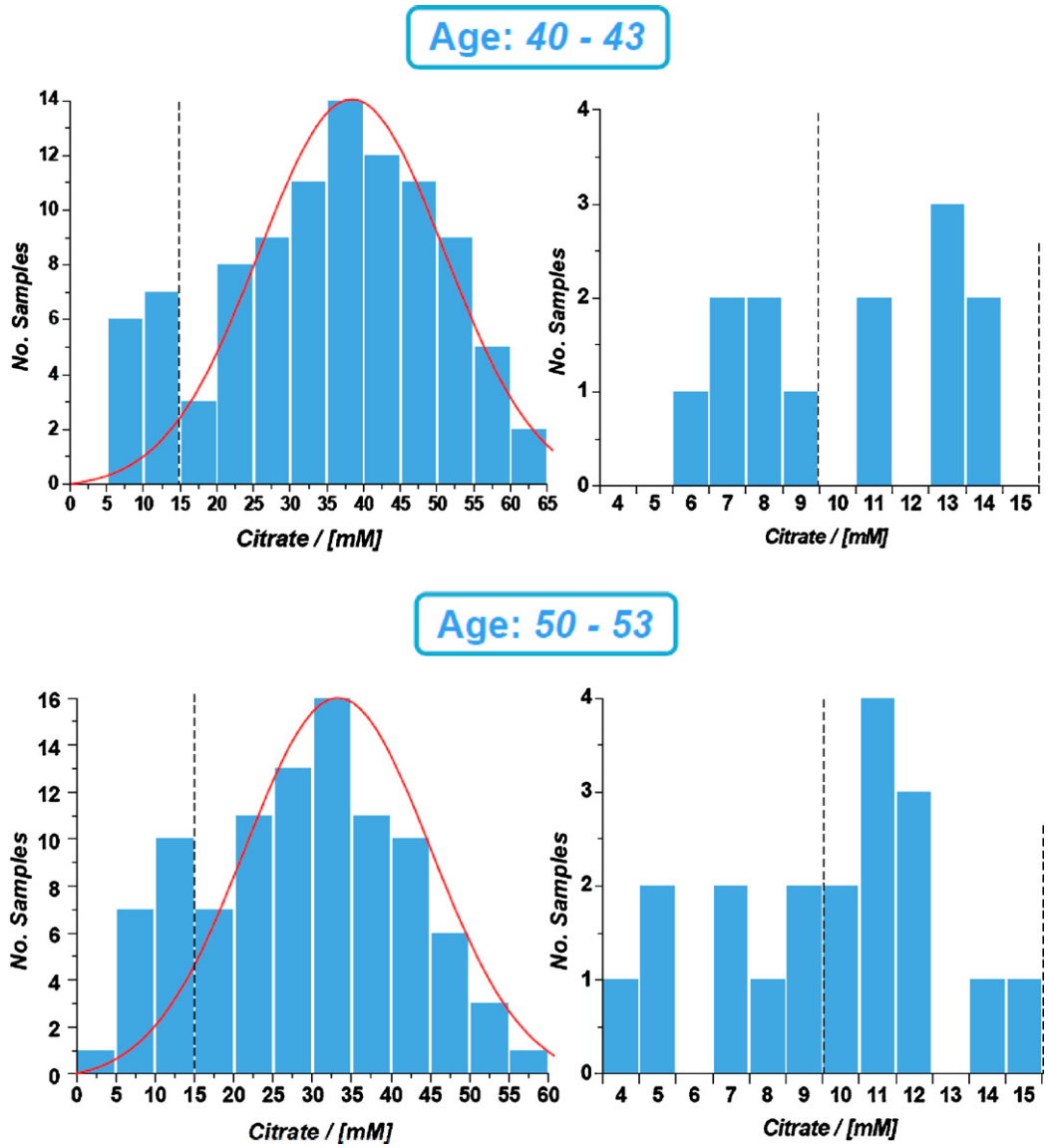


Fig. 5. Distribution of citrate concentrations determined by the europium luminescence assay for 97 (upper) and 96 (lower) seminal fluid samples in each of the stated age ranges; expansions of citrate levels that fall in the range (between and including) 4–15 mM are shown on the right. The curves show calculated Gaussian distributions for the 84 samples in the 40–43 range (mean 38.8 mM, half-width/half-max. 25) and the 77 samples in the 50–53 range (mean 32.8 mM half-width/half-max. 22.5).

inal fluid to be assessed rigorously, as a *screening* biomarker for prostate cancer.

4. Conclusions

An analytical method has been developed and validated allowing citrate levels in low volume seminal fluid samples to be assayed. In parallel, a compact time-gated instrument has been built that allows readings to be taken rapidly; sample dilution into a buffer containing lactate is the only pre-treatment step that is required. The distribution of citrate values in seminal fluid recorded for two cohorts of men indicate that citrate levels do not change significantly with age; values measured fell within one SD.

The slight increase in the proportion of low ($\leq$15 mM) citrate values in the aged 50–53 cohort could be ascribed to increased numbers of samples from men with prostatitis, but could in theory also be associated with patients with prostate adenocarcinoma. Therefore, a larger clinical study is warranted to allow the utility of citrate assays in seminal fluid to be defined. The facility and rapidity of the analytical method defined herein may offer an opportunity for this to be examined in a large-scale screening programme for prostate cancer in the over-50 age group. This needs to be coupled to a definition of the nature and extent of any prostatic inflammation, that is normally diagnosed following urinalysis and rectal examination [29].

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