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Ultra high field NMR spectroscopic studies on human seminal fluid, seminal vesicle and prostatic secretions

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Abstract: Ultra high field ^{1}H-NMR spectroscopic methods have been used to analyse the composition of seminal fluid and its component secretions, prostatic and seminal vesicle fluids from normal human subjects and those with vasal aplasia and non-obstructive infertility. The ^{1}H-NMR spectrum of whole seminal fluid is extremely complex and many resonances are extensively overlapped in single pulse spectra even when measured at 600 or 750 MHz ^{1}H resonance frequency. A combination of 2-D ^{1}H-NMR methods (including J-Resolved and various ^{1}H homonuclear correlation and ^{1}H–^{13}C heteronuclear correlation techniques) were applied at 600 or 750 MHz in order to extensively assign the signals from the organic components of seminal fluid. Prostatic fluid (PF) gives a much less complex metabolite profile than whole seminal fluid and can be completely analysed using 500 MHz ^{1}H-NMR spectroscopy. The ^{1}H-NMR spectra of prostatic fluid are dominated by signals from citrate, spermine and *myo*-inositol, whereas the spectra of seminal vesicle fluid (SVF) show extensively overlapped signals from complex peptide mixtures together with strong signals for glycerophosphocholine (GPC) and lactate. Whole seminal fluid is a combination of the PF and SVF constituents together with further substances that appear after mixing due to the operation of PF enzymes on SVF, e.g. peptidase activity causes rapid cleavage of peptides to amino acids and GPC is hydrolysed to choline, glycerol and inorganic phosphate. It is also shown that vasal aplasia leads to highly characteristic abnormal metabolite profiles in seminal fluid that can be readily observed in single-pulse 500 and 600 MHz ^{1}H-NMR spectra. Measurement of the molar citrate to choline, or spermine to choline ratios in seminal fluid both show differences of 2 orders of magnitude between vasal aplasia (greater for both ratios) and non-obstructed infertile patients. This work gives an indication of the potential of high field ^{1}H-NMR spectroscopy in the investigation and assessment of the secretory functions of the male genital tract and the evaluation of the infertile male subject.

Keywords: *750 MHz ^{1}H-NMR; seminal fluid; citrate; spermine;* myo-*inositol; choline; vasal aplasia; azoospermia.*

Introduction

Seminal plasma contains high concentrations of a diverse range of low MW weight organic compounds and inorganic ions, some of which, e.g. Zn^{2+}, many amino acids, spermine and citrate are present in concentrations up to 2 orders of magnitude higher than any other mammalian body fluid [1]. The protein composition of seminal fluid (SF) is also complex and in a recent review [2] some 17 different secretory proteins were identified in the prostate and seminal vesicles using immunohistochemical methods. SF is predominantly formed by the combination of the secretions of the prostate and the seminal vesicles, and to a lesser extent the testis, the composition of each secretion being biochemically distinct. Prostatic fluid (PF) contains uniquely high levels (>10 mM) of citrate, polyamines and *myo*-inositol [1, 3–5] and up to 7 mM zinc, i.e. about 20 to 30 times plasma levels, in a chemically ill-defined form [6–8]. Seminal vesicle fluid (SVF) contains high but variable levels of fructose, and glycerophosphocholine [9] which is more typically found in intracellular locations [10]. Each of the contributory fluids is biochemically active and a series of enzymatic reactions are initiated on mixing (prior to ejaculation) which results in alterations to the biochemical composition that assist fertility. Despite the importance of the dynamic interactions of the components of SF in the preservation of fertility, the functional

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biochemistry of the fluids are poorly understood due to the severe analytical difficulties encountered in this complex matrix.

PF forms the first component of the ejaculate [7, 8] together with the spermatocytes and it is likely that the fluid is in some way essential for short term gamete survival. Along with low MW components mentioned above, PF contains a number of unique enzymes: prostatic acid phosphatase (tartrate labile), and prostate specific antigen. The prostate is a common site of disease in elderly men [11–13]. The concentrations of many of the seminal fluid constituents have been shown to vary in different prostatic diseases and these have been reviewed by Daniels and Grayhack [8, 14]. The concentrations of the polyamines (putrescine, spermidine and spermine) in SF also vary with sperm motility [9] and fertility [15]. Zn^{2+} is present in high concentrations in the prostate, PF and spermatozoa and is thought to have some antimicrobial activity [16] as well as being important in the maintenance of the quaternary structure of the sperm chromatin [17]. The role of citrate has been assumed to be as a ligand for zinc [17] although the direct evidence for this is weak given the difficulty of analysing the speciation of metal ions in such a complex matrix. It is doubtful that citrate could act as an energy source for the spermatozoa because of poor absorption due to its triple negative charge at physiological pH. The mechanisms of secretion by the prostate of the unique combination of low MW substances into the PF or indeed the exact biological significance of the unusual metabolite composition are poorly understood. The biochemical interactions of low MW compounds that occur when prostatic and SVFs are mixed also remains largely uninvestigated due to the severe analytical problems imposed by the intact matrix. There is therefore a need to develop and apply novel analytical methods for the investigation of these fundamental dynamic processes.

Although not of immediate relevance to studies of fertility, analysis of PF is also important because of insights it may provide into changes in the prostate in disease states. There is much interest in the prostate as it is the source of much morbidity and mortality; 10% or more of men will require an operation for prostatic enlargement at some time in their life [11], and prostate cancer is the most common cause of internal cancer in men in the United States [12]. The incidence of prostate cancer increases with age and some of this increase may reflect improving longevity [13]. Therefore, prostate cancer is likely to become an even greater problem with current trends towards increasing life-span and there is an urgent need to develop new tools for the investigation and diagnosis of this widespread problem.

Seminal vesicle fluid (SVF) forms the later part of the ejaculate. Obtaining pure samples of this can only be achieved by invasive means (surgery or aspiration with a wide bore needle as the fluid is too viscous to be extracted through standard narrow bore needles). With the difficulty in obtaining SVF, it then becomes necessary to maximize the information obtained through the use of modern analytical methods (such as non-destructive NMR spectroscopy), which are not restricted to an *a priori* selection of analyte and which can additionally give information on molecular dynamics, protein-binding and chemical exchange processes.

High resolution ^{1}H-NMR spectroscopy of biofluids has emerged as a powerful modern tool with which to explore the abnormal biochemical processes that result from toxin-induced tissue damage [18–20], inborn errors of metabolism or other metabolic diseases [21–26]. We have previously published some preliminary data on the 400 MHz ^{1}H-NMR spectrum of SF but because of the complexity of the fluid only a partial assignment was possible at this field strength [27]. In a recent study using 200 MHz ^{1}H-NMR spectroscopy of SF only four metabolites were assigned even with the aid of two-dimensional correlation techniques [28], but the authors suggested that their results gave additional practical clinical information. This paper identified glycerophosphorylethanolamine (GPE) as a diagnostic marker of semen quality, particularly when related to GPC levels [28]. GPE had not been identified in seminal plasma in any significant amounts in previous conventional studies and the NMR evidence for this assignment was inconclusive from the 200 MHz results presented. A major aim of this study was to assign as far as possible the ^{1}H-NMR spectra of seminal plasma with the aim of identifying metabolites likely to give useful information on semen quality and function. Therefore, a series of ultra high field ^{1}H-NMR spectroscopic studies have been undertaken to

elucidate further the biochemical composition and dynamics of whole human SF and its component fluids in normal and diseased states. In this report the detailed assignment of the one and two dimensional ^{1}H- and ^{13}C-NMR spectra of SF, PF and SVF are presented in which a total of 37 metabolites have been identified. The diagnostic potential of high resolution ^{1}H-NMR spectroscopy in the assessment of male reproductive physiology and fertility is also illustrated.

Methods and Materials

Samples and preparation

SF samples were collected routinely from non-obstructed infertile patients attending an infertility clinic and presenting with the following conditions: with sperm antibodies (n = 3), normal fertile subjects (n = 3) and those with vasal aplasia (i.e. obstruction of the *vas deferens* such that the secretion of the seminal vesicles was blocked, n = 13). Each SF sample was allowed to stand at room temperature for 30 min post-ejaculation (during which time the SF gel is liquefied by endogenous enzymatic activity and the viscosity is greatly reduced) the pH was then measured. The specimens were viewed by light microscopy to determine sperm count, concentration and motility; and were also tested for sperm antibodies. The remaining SF was frozen at −70°C until the NMR measurements were made. Prostatic secretions (n = 10) were obtained by prostatic massage and SVF (n = 3) was collected at major retropubic surgery. To reduce viscosity, all samples were diluted by 50% with D_2O containing 0.5 mM sodium 3-(trimethylsilyl-2,2,3,3-$^2H_4$)-1-propionate (TSP, δ0).

Proton NMR spectroscopy

Single pulse ^{1}H-NMR spectra were obtained using a Varian VXR600s spectrometer operating at 599.95 MHz ^{1}H resonance frequency or a JEOL GSX500 spectrometer operating at 500.13 MHz ^{1}H resonance frequency. All spectra were measured at ambient probe temperature. For each sample at 600 MHz, 64 free induction decays (FIDs) were collected into 32,768 computer data points with a spectral width of 8000 Hz. In single pulse experiments 30° pulses were used with an acquisition time of 2 s and a delay between pulses of 2.7 s. The water signal was suppressed by applying a gated selective secondary irradiation field at the water resonance frequency during the delay between pulses (gated off during acquisition). Prior to Fourier transformation (FT), an exponential apodization function was applied to the FID corresponding to a broadening of 0.2 Hz. Selected samples were also measured on a Bruker AMX750 spectrometer operating at 750.14 MHz with parameters adjusted appropriately and the water signal was suppressed using a 1-D NOESY presaturation scheme. Where possible signal assignments were made by reference to the literature and standard additions of candidate compounds, but, given the complexity of the ^{1}H-NMR spectra of the SFs a combination of 2-D NMR spectroscopic methods were used on selected samples to aid signal assignment and the NMR data analysed interactively in order to assign the spectra as fully as possible (see below).

Homonuclear ^1H 2-D J-resolved spectroscopy (JRES)

600 MHz and 750 MHz ^{1}H-JRES [29] spectra of seminal plasma were obtained by sequential repetition of the pulse sequence:

$$[D - 90° - t_1 - 180° - t_1 - \text{collect FID for time } t_2],$$

where D = 2.7 s, and t_1 was an incremented variable delay. A secondary irradiation field was applied at the water resonance frequency during delay D which was gated-off during the application of the pulse sequence and acquisition. The F_2 (chemical shift) domain was collected into 8192 computer points with a spectral width of 6000 Hz (600 MHz measurements) or 10000 Hz (750 MHz measurements), and the F_1 (J-coupling) domain covered 30 Hz with 64 increments of t_1. Typically 16 transients were collected for each t_1 increment in the JRES experiments. Prior to the double FT, the data were apodized by means of a sine-bell function in t_1 and t_2. The spectra were tilted by 45° to provide orthogonality of the chemical shift and coupling constant axes. Following a magnitude calculation, spectra were displayed both in the form of contour plots and skyline F_2 projections.

^1H–^1H correlation spectroscopy (COSY-45)

600 MHz COSY45 [30] spectra were collected using the following pulse sequence:

$[D - 90° - t_1 - 45° -$ collect FID for time $t_2]$,

where D was a 2.7 s T_1 relaxation delay, and the period t_1 was incremented to allow exchange of magnetization of spin–spin coupled protons. Data were collected into 2048 data points in F_2 using 16 scans per increment with a spectral width of 6000 Hz and 512 increments of F_1 were zero-filled to 2048 before FT. The FID was weighted using a sinebell function in t_1 and t_2 prior to FT and magnitude spectrum calculation.

^1H–^1H total correlation spectroscopy (TOCSY)

In order to confirm the ^{1}H-NMR assignments, particularly of the chains of coupled protons in lysine and arginine, 600 MHz TOCSY spectra [30, 31] were measured using the following pulse sequence:

$[D - 90° - t_1 -$ (spin lock) $-$ acquire FID for time $t_2]$.

Solvent saturation was achieved using irradiation at the water resonance during the relaxation delay D = 2.7 s. The spectra were collected in the phase-sensitive mode using TPPI [31]. The MLEV17 pulse sequence [31] was used for the spin-lock. The spectral width was 8064 Hz with data collected into 4096 time domain points. Typically 512 incements of t_1 were measured with 16 FIDs per increment, the data being zero-filled to 1024 in F_1 prior to FT. A sinebell squared apodization function was applied in both time domain FIDs prior to FT.

^1H–^{13}C heteronuclear multiple quantum correlation spectroscopy (HMQC)

600 MHz ^{1}H–^{13}C HMQC [30, 32–34] spectra were collected with an inverse-detection probe using a BIRD [33] pulse to suppress ^{1}H signals for protons connected to ^{12}C and with composite pulse ^{13}C decoupling during acquisition. The pulse sequence is:

$[D - 90°_{1_H} - d_2 - 90°_{13_C} - t_1 - 180°_{1_H} - t_1 - 90°_{13_C} - d_2 -$ acquire FID for time $t_2]$,

where D is a relaxation delay of 2.1 s, d_2 is a refocusing delay equal to 1/2 $^1J_{C-H}$, t_1 is an increment delay and average value of $^1J_{CH}$ of 140 Hz was used to determine d_2.

Results and Discussion

Assignment of the ^1H-NMR spectra of seminal fluids

High resolution NMR spectrometers that operate at 750 MHz ^{1}H resonance frequency (17.63 Tesla) have recently become available. However even at this field, single pulse or spin echo spectra of biological fluids such as blood plasma show considerable signal overlap over a wide chemical shift range, particularly in the 'aliphatic' region of the spectrum from δ 0.7–4.7 [35, 36]. This overlap phenomenon is referred to as 'chemical noise' [21] and occurs where there is multiple superimposition of peaks from low concentrations of metabolites that are within the NMR detection range (i.e. resulting in digitized signals), but which cannot be identified individually using currently available multiple pulse sequences. This is because their resonances overlap so extensively that only a broad envelope results in the single pulse spectrum and, individually, the levels of single metabolites may approach the lower limits of NMR detection (currently <10 nM ml^{-1}) rendering them difficult to observe in less sensitive 2-D experiments. This poses a problem both of signal assignment for many endogenous biomolecules and a possible source of error in quantitation of single pulse spectra in which the chemical noise is greater in certain chemical shift ranges than others [21]. The complexity of 750 MHz ^{1}H-NMR spectra of SF obtained from a healthy individual is illustrated in Fig. 1 with many of the signals assigned (see below). The sample illustrated like the others in this study, was subjected to liquefaction for 30 min prior to freezing and diluted by 50% with D_2O prior to NMR measurement. Fresh undiluted seminal fluid gives rise to ^{1}H-NMR spectra with very broad and poorly resolved signals due to the presence of high concentrations of peptides (which are cleaved to amino acids by endogenous peptidase activity) and the high viscosity of the matrix with a consequent general shortening of proton T_2 relaxation times (data not shown). The biochemical complexity of the fluids revealed at 750 MHz (Fig. 1) indicates that NMR assignments and quantitative NMR measurements performed on seminal fluid at lower field strengths are likely to be very difficult and in some cases incorrect assignments appear to have been made [27, 28]. In a recent study on seminal fluid performed at 200

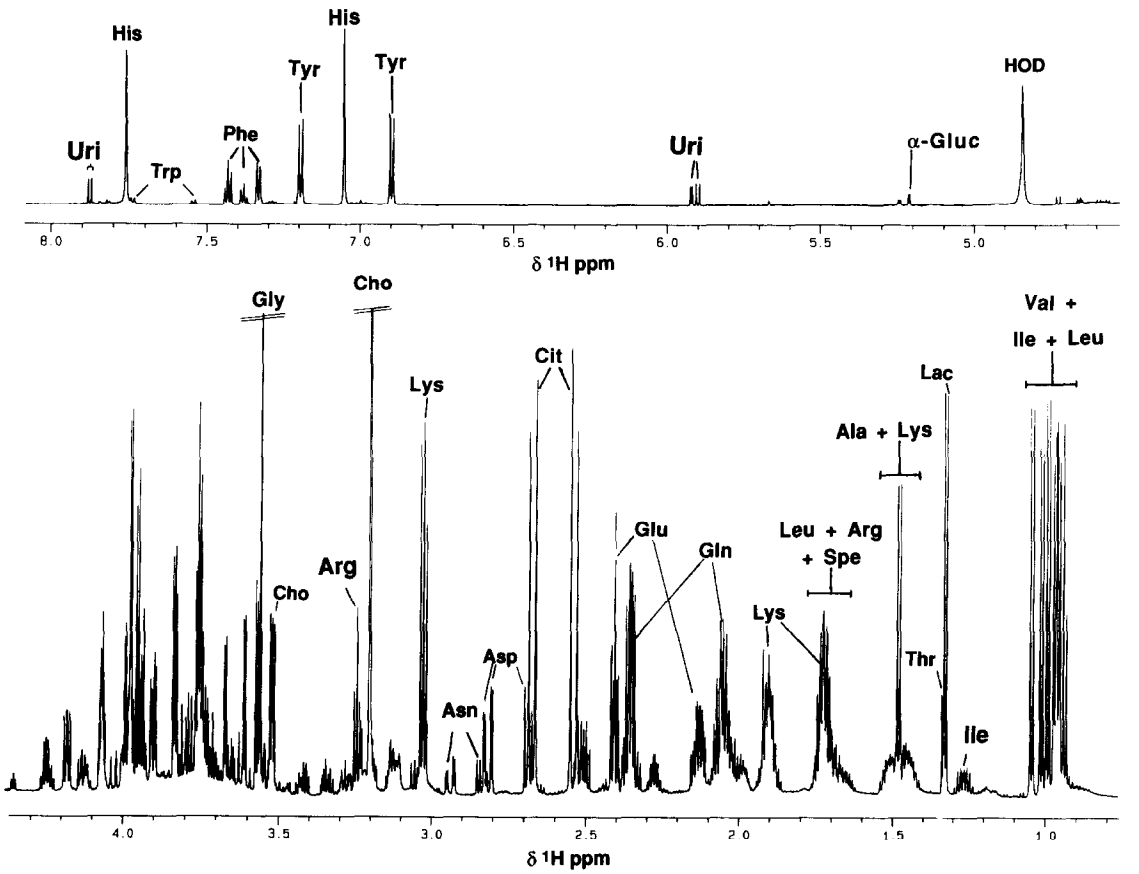

Figure 1
Single-pulse 750 MHz ^{1}H-NMR spectrum of human seminal plasma from a normal subject diluted 50% with D_2O. Lower trace from δ 0.65 to 4.5, upper trace from δ 4.5 to 8.1. Key: Val, valine; Ile, isoleucine; Leu, leucine; Lac, lactate; Thr, threonine; Lys, lysine; Ala, alanine; Arg, arginine; Spe, spermine; Glu, glutamate; Gln, glutamine; Cit, citrate; Asp, aspartate; Asn, asparagine; Cho, choline; Gly, glycine; α-gluc, H_1 anomeric proton of α-glucose; Tyr, tyrosine; Uri, uridine; His, histidine; Phe, phenylalanine, Trp; tryptophan.

MHz, it was not possible to distinguish (and hence assign) the various N-methyl containing species and unfortunately GPC was assigned as glycerophosphorylethanolamine (although this component does not give rise to a singlet resonance) and choline as GPC with consequential incorrect biochemical interpretation. Measurement at such low field strengths would also cause great difficulty in obtaining accurate integral values because of the extent of overlap of the metabolites. We have previously considered the arguments concerning accuracy of quantitation in metabolite measurements at lower field [21, 26] but clearly the higher the field strength used the greater the sensitivity and spectral dispersion and hence more resonances are resolved resulting in better accuracy reliability. The biochemical information content of an ultra high field biofluid NMR spectrum is intrinsically much greater than that obtained at lower field, and therefore, efficient solutions to problems raised by chemical noise and spectral assignments for minor metabolites are worth pursuing. Many of the major metabolites can be assigned directly in 500, 600 or 750 MHz single pulse spectra of control SF, e.g. lactate, alanine, choline, valine, citrate, the aromatic amino acids, histidine, uridine and formate. However, there are a multitude of signals from compounds with more complex or heavily overlapped spin systems that are much more difficult to assign by simple consideration of single pulse spectra even when measured at 750 MHz. Some signals also appear to have anomalous chemical shifts when compared to standard aqueous solutions measured at the same pH. The signals from glutamate (particularly the γCH_2) appears to high frequency of the γCH_2 of glutamine possibly due to the formation of a stable carbamate complex by reaction with bicarbonate under the alkaline

conditions (≥pH 7.5) that generally prevail in seminal fluids. The phenomenon of spontaneous carbamoylation has also been reported for glutamate, alanine and taurine in NMR studies on carbonate/bicarbonate neutralized tissue extracts [37].

The application of the 2-D JRES experiment on human SF either at 600 or 750 MHz results in a dramatic simplification of the spectrum due to the dispersion of the chemical shift and coupling constant data in two orthogonal frequency domains. We have previously discussed the advantages of applying very high field JRES spectroscopy for the analysis of other biofluids such as urine and blood plasma [35, 36]. JRES spectroscopy enables the complex overlapped resonances in some of the chemical shift ranges (especially from δ 3 to 4) to be more completely resolved. Figure 2 shows the aliphatic region of the 600 MHz ^{1}H-JRES spectrum of SF from an azoospermic subject (δ 0.9–4.6). The skyline F_2 projection through the JRES map results in a greatly simplified spectral profile of the effectively ^{1}H-decoupled proton spectrum of the motionally unconstrained low MW metabolites in seminal plasma. Signals from any small molecules that are extensively protein-bound or involved in chemical exchange reactions on the intermediate NMR timescale will also be severely attenuated because of their short T_2 relaxation times. The JRES experiment where applied to biofluid analysis offers high efficiency with respect to the time needed for the acquisition of spectra with good signal to noise ratios (≤20 min) together with outstanding resolution and sensitivity. The use of JRES experiments for spectral simplification of seminal plasma would be indicated in cases where abnormal sugar or polyol components are being examined as resonances from these compounds largely occur in the complex spectral region from δ 3 to 4. Indeed this region of the spectrum of any biofluid is perhaps the most complex (i.e. richest in biochemical information) and has hitherto been closed to detailed NMR study because of spectral interference. JRES spectroscopy offers an efficient means of simplifying this region of the spectrum. At 750 MHz, JRES spectroscopy is even more effective because of the greater sensitivity and dispersion [35] as shown for SF in Fig. 3 in relation to the single pulse spectrum of the same sample. The 750 MHz JRES spectrum

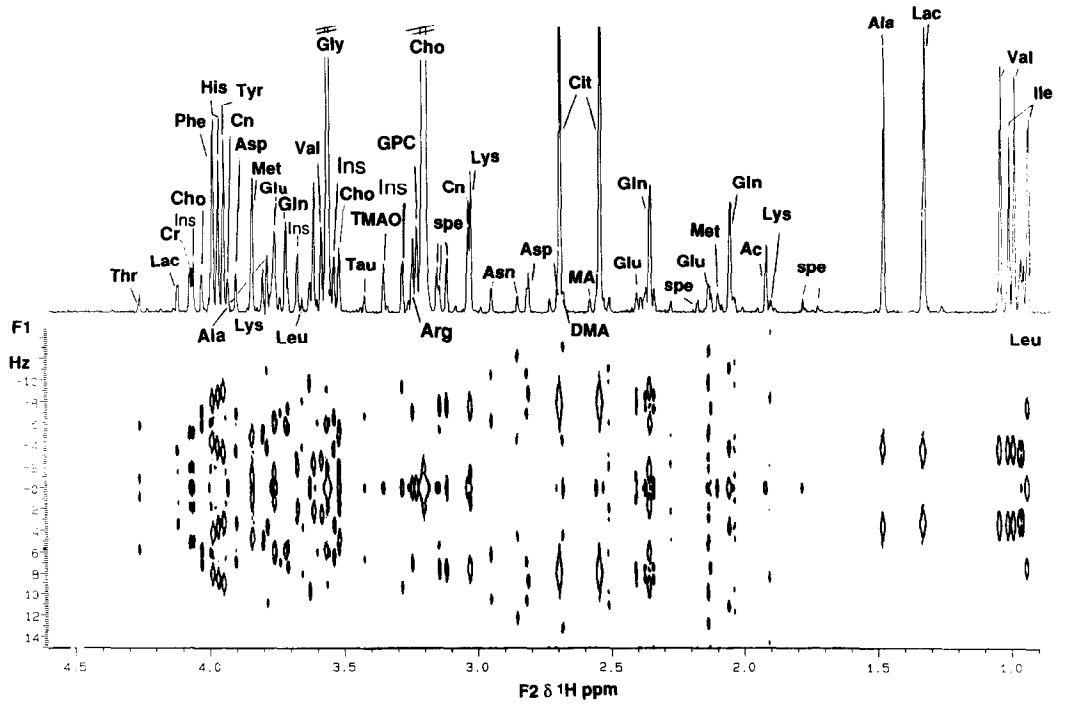

Figure 2
600 MHz JRES ^{1}H-NMR spectrum of the aliphatic region of control human seminal fluid from δ 0.9 to 4.6, showing contour plot and skyline F_2 projection. Key as in Fig. 1 plus, Ac, acetate; Met, methionine; Pyr, pyruvate; MA, methylamine; DMA, dimethylamine; Asp, aspartate; Cn, creatine; TMAO, trimethylamine-N-oxide; Ins, *myo*-inositol; Tau, taurine; GPC, glycerophosphorylcholine.

shown in Fig. 3(a) shows strong contributions from the heavily-coupled (and hence much weaker) ^{1}H multiplets of lysine, arginine, glutamine and glutamate; these are not observed or only seen with difficulty at 600 MHz (e.g. Fig. 2). The one-dimensional and JRES spectra of this SF sample also show the presence of a high concentration of ethanol, presumably of exogenous origin due to consumption by the subject prior to sample collection. This is a particularly interesting observation as to our knowledge ethanol has

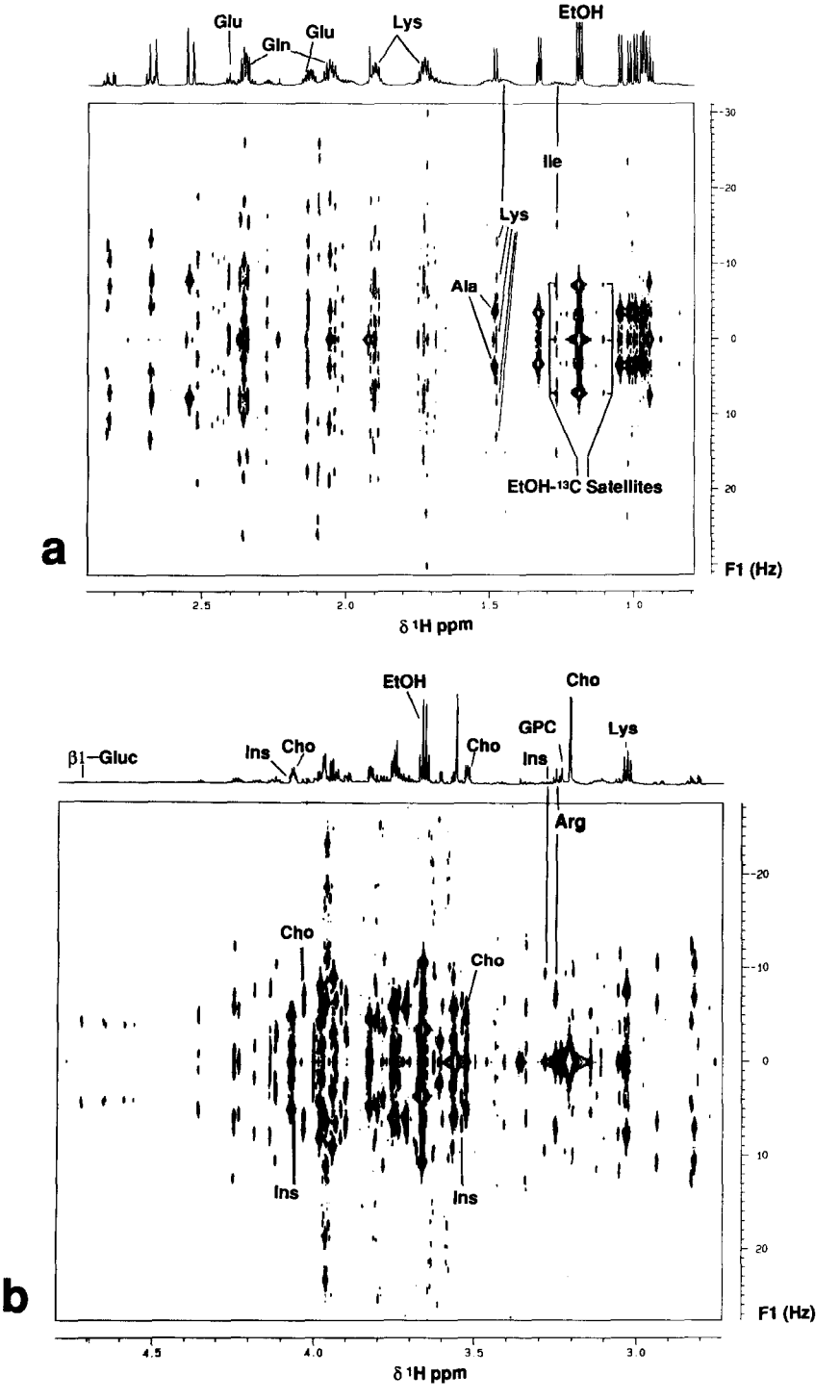

Figure 3
750 MHz JRES ^{1}H-NMR sub-spectra of the aliphatic region of control human seminal fluid shown in relation to 750 MHz single-pulse spectra of the same sample from (a) δ 0.8–2.9 and (b) δ 2.7–4.8. Key as in Fig. 1 plus EtOH, ethanol.

not previously been detected in SF. The JRES spectrum also shows both ^{13}C satellite triplet peaks of the ethanol methyl signal ($^1J_{CH}$ = 144 Hz). This is important as it indicates that when spectra of biofluids are measured at very high field (with the consequent increase in sensitivity) it would easily be possible to mistake ^{13}C satellites of high concentration metabolites as signals from minor metabolites. The portion of the 750 MHz JRES spectrum shown in Fig. 3(b) shows the resolution of the peaks for choline, GPC, arginine (N-CH_2) *myo*-inositol (H_5) that were misidentified in a previous 200 MHz ^1H-NMR study of SF [28]. The ethanol CH_2 signal is also clearly shown although its ^{13}C satellites are obscured by other resonances.

The aliphatic region of the 600 MHz ^1H–^1H COSY45 spectrum of human SF is shown in Fig. 4 with signal assignments. This type of spectrum results in off-diagonal cross peaks for spin–spin coupled protons and, because the spectrum is now dispersed in two chemical shift domains it minimizes the peak overlap for complex spin systems. COSY is a less time efficient technique than JRES because of the larger number of F_1 experiments needed, and this also compromises the digital resolution of the COSY experiment compared to JRES. However, COSY is an essential tool in biofluid metabolite assignment as it is used in combination with JRES to establish the connectivity patterns of the spin systems revealed by JRES.

For the unequivocal identification of metabolites in which spin coupling exists along chains of CH_2 or CH protons it is highly advantageous to measure the total correlation or TOCSY spectrum of the biofluid. This experiment results in off-diagonal peaks for all the chemical shifts along an unbroken chain of proton–proton couplings as illustrated in Fig. 5. The TOCSY experiment was particularly useful for showing the connectivity maps for arginine and lysine which are extensively overlapped with each other and other metabolites. The full connectivities of the spin systems for both of these compounds are shown in Fig. 5.

The inverse-detected ^1H–^{13}C heteronuclear multiple quantum coherence transfer experiment (HMQC) provides second-dimensional dispersion in the carbon frequency domain with its much greater chemical shift range and is illustrated for SF in Figs 6 and 7. In this example a BIRD nulling [32] sequence was used to eliminate the signals from protons on

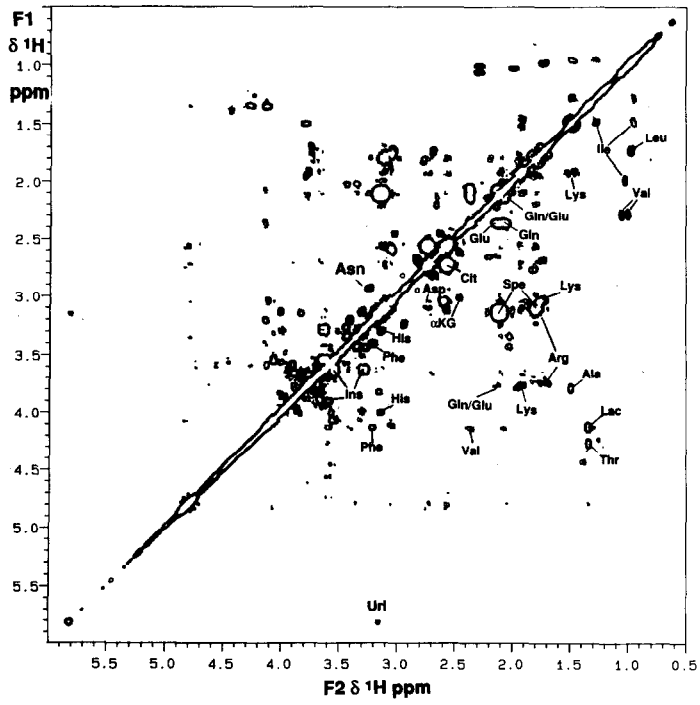

Figure 4
600 MHz COSY45 ^1H-NMR spectrum of a control human seminal fluid from δ 0.5 to 6.0, abbreviations as listed in previous figures; Uri, indicates the position of the cross peak for the H_1 to H_2 ribosyl connectivity of uridine.

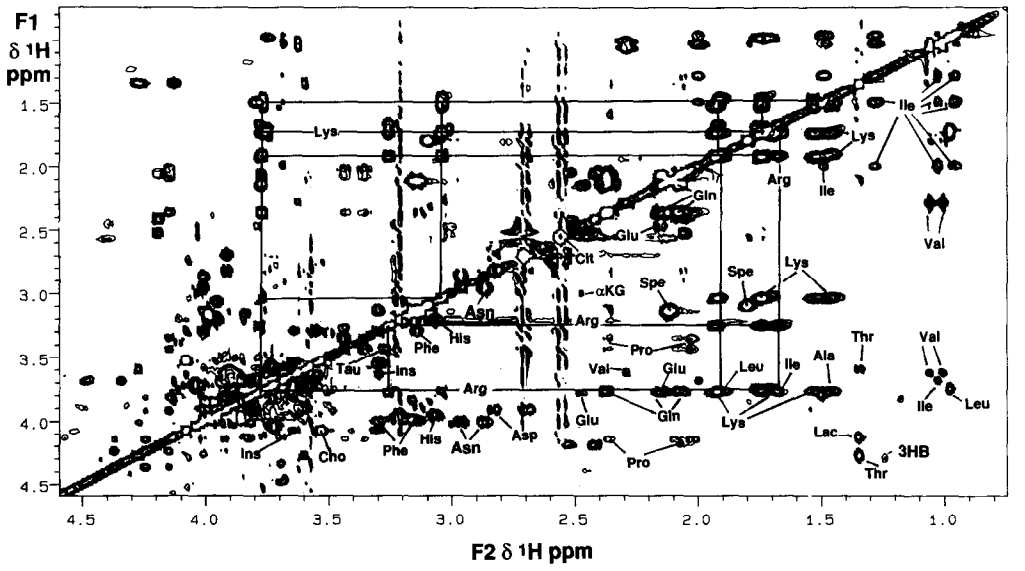

Figure 5
600 MHz TOCSY ^{1}H-NMR spectrum of a control human seminal fluid from δ 0.75 to 4.6, abbreviations as listed in previous figures plus 3HB, 3-D-hydroxybutyrate, Pro, proline, α-KG, α-ketoglutarate. The complete connectivities for lysine and arginine are shown above and below the diagonal, respectively.

^{12}C nuclei (i.e. approx. 99% natural abundance) and broadband carbon decoupling was used to obtain singlet cross peaks in the 2-D contour plot. Many of the major amino and organic acid signals can be assigned based on their ^{1}H chemical shifts in the 2-D experiments but some are much more easily distinguished and separated in the heteronuclear 2-D HMQC experiment, e.g. the spermine CH_2N groups, that from the CH_2N group of arginine and the choline $N^+(CH_3)_3$ group (Fig. 6). We have noted some variation in the exact ^{13}C-NMR shifts in HMQC spectra of SF which are consistent with the normal pH variations in that fluid. The combination of the various homonuclear and heteronuclear techniques has resulted in the assignment of most of the major and many of the minor resonances in the SF spectrum and, therefore, provides the basis of the use of NMR for probing biochemical interactions of the SF components in the intact matrix or the use of NMR as a diagnostic probe of altered male reproductive gland function (see below). The assignments are marked in detail on Figs 2–7 and those data reported in full in Table 1. In contrast to previous workers using 200 MHz spectroscopy to analyse seminal fluids [28], there was no NMR evidence for the presence of GPE in any of the seminal fluids examined.

Comparison of the single-pulse ^{1}H-NMR spectra of prostatic and seminal vesicle fluids

The typical NMR spectra of expressed PF and SVF (collected at surgery) of the same man are compared in Fig. 8. There are significant differences in the ^{1}H-NMR spectroscopic metabolite profiles of the two fluids. The SVF (which forms 50–85% of the total SF secretion) contains no citrate or spermine, but has large amounts of GPC, lactate and has signals from a variety of peptides which are ultimately cleaved to amino acids when the fluids are mixed due to the endogenous peptidase activity (prostate specific antigen and prostatic acid phosphatase) of the prostatic secretion. The chemical shift of the N-methyl signal of GPC in SVF is nearly identical to that found in phosphate-buffered saline at pH 7 (δ3.39). However, on mixing SVF with PF there is an immediate upfield shift of this signal to 3.29 ppm rendering resolution from the H5 *myo*-inositol signal impractical in single pulse spectra of SF except when measured at the highest possible field strength (Fig. 1). There is also a rapid enzymatic hydrolysis of GPC to choline (90% conversion in <5 min at 37°C) that occurs on mixing SVF and PF which is catalysed by endogenous esterases in the PF with a resulting upfield shift of the N-methyl signal to 3.21 ppm as the choline is released

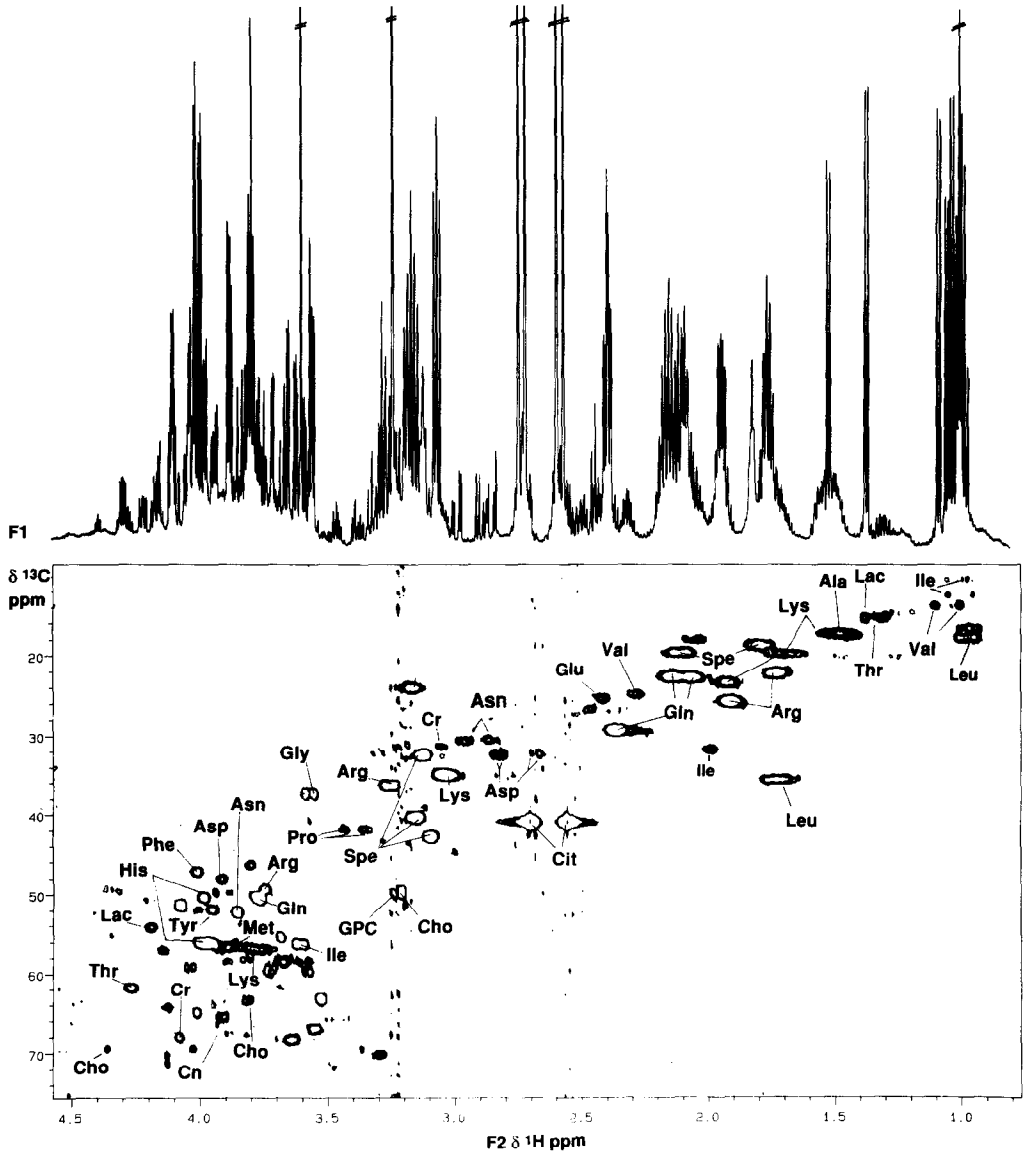

Figure 6
600 MHz ^{1}H–^{13}C HMQC spectrum (contour plot) of the aliphatic region (δ 0.75–4.6) of control seminal fluid (same sample as shown in Fig. 5) in relation to its single pulse spectrum (above). Key as for previous figures.

(data not shown). Thus GPC provides only a minor contribution to the SF spectrum when the SF samples from normal or azoospermic patients are not measured immediately. The timed GPC–choline ratio can be measured following collection of SF and this gives a method for monitoring the enzymic activity and relative contributions of the prostatic and seminal vesicle contributions to the final fluid which may have potential in the differential diagnosis of male reproductive tract disorders. The dynamic interactions that occur on mixing SVF and PF, particulairly those related to esterase cleavage of GPC, changes in metal complexation and enzyme-mediated peptide cleavage are currently under investigation.

^{1}H-NMR spectroscopy of seminal plasma from patients with infertility conditions

Previous work has suggested a value for NMR assessment of seminal plasma in infertility using ^{31}P-NMR [38] which is only about 6% as sensitive as ^{1}H-NMR. Furthermore, there are only a few phosphorus-containing metabolites that are present in significant concentrations either in body fluids or cells and therefore ^{31}P-NMR is intrinsically more limited than ^{1}H-NMR spectroscopy as a probe

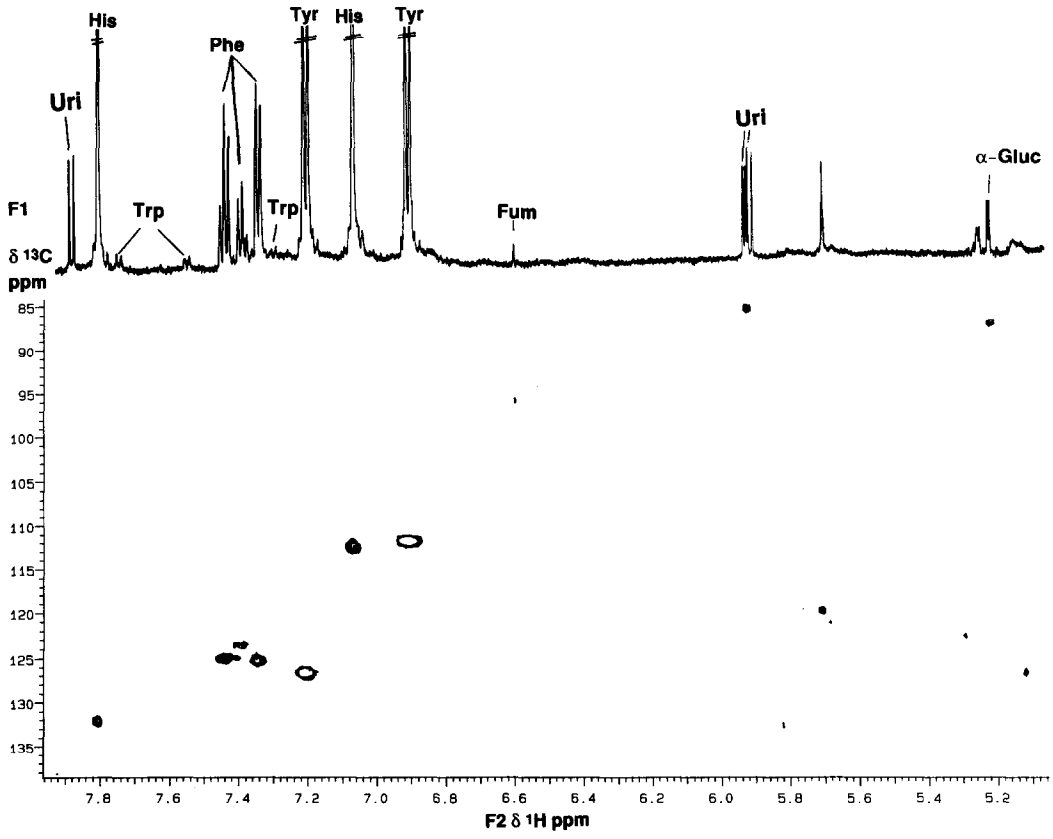

Figure 7
600 MHz ^{1}H–^{13}C HMQC spectrum (contour plot) of control seminal fluid (same sample as shown in Fig. 6) from δ 5.05 to 7.96 in relation to its single pulse spectrum (above). Key as for previous figures. Note that not all signals seen in the single pulse spectrum are strong enough to be observed in the HMQC spectrum.

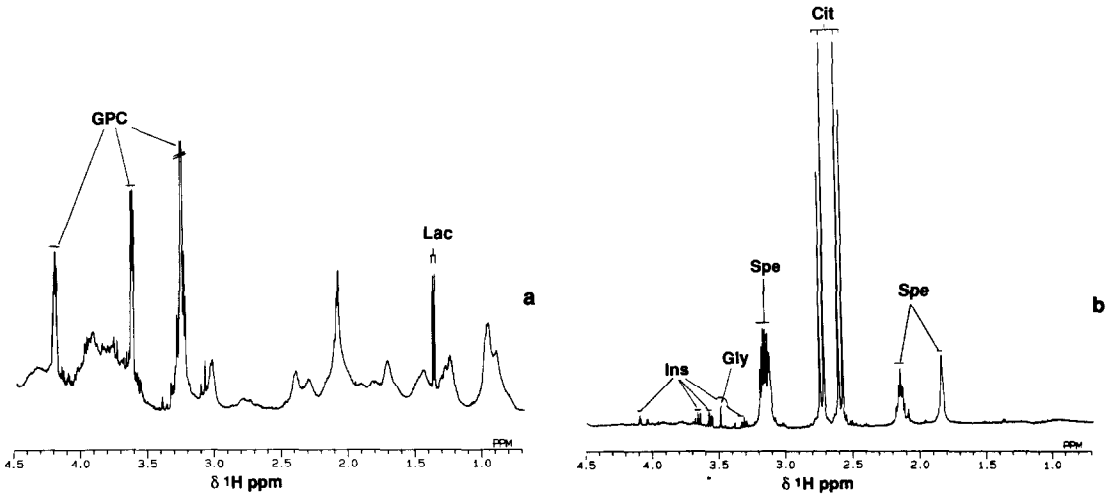

Figure 8
Single-pulse 500 MHz ^{1}H-NMR spectra of (a) control seminal vesicle fluid taken at prostatic surgery and (b) control human prostatic fluid collected by the Stamey procedure. Note the contrasting metabolite profiles of the two fluids. The broad unresolved resonances seen in the seminal vesicle fluid (from δ 0.9 to 2.5 and 3.7 to 4.6) are due to the superimposition of the signals from a variety of peptides and proteins.

Table 1
Metabolite resonance assignments in 600 and 750 MHz ^{1}H-NMR spectra of human seminal fluid at pH 7.5

Shift (δ)	Multiplicity	Compound	Assignment
0.93	t	isoleucine	δ-CH_3
0.96	d	leucine	δ-CH_3
0.97	d	leucine	δ-CH_3
0.97	d	valine	CH_3
1.00	d	isoleucine	β-CH_3
1.18	t	ethanol	CH_3
1.20	d	3-D-hydroxybutyrate	γ-CH_3
1.28	m	isoleucine	γ-CH_2
1.32	d	lactate	CH_3
1.30	d	threonine	γ-CH_3
1.47	m	isoleucine	γ-CH_2
1.48	m	lysine	γ-CH_2
1.46	d	alanine	CH_3
1.66	m	arginine	γ-CH_2
1.69	m	lysine	δ-CH_2
1.70	m	leucine	β-CH_2, γ-CH
1.78	m	spermine	$NH_2CH_2(\underline{CH_2})_2$
1.91	s	acetate	CH_3
1.91	m	lysine	β-CH_2
1.91	m	arginine	β-CH_2
1.96	m	isoleucine	β-CH
2.03	m	proline	γ-CH_2
2.05	m	3-D-hydroxybutyrate	α-CH_2
2.06	m	proline	β-CH_2
2.06	m	glutamine	β-CH_2
2.13	s	methionine	CH_3
2.14	m	glutamate*	β-CH_2
2.16	m	methionine	β-CH_2
2.17	qu	spermine	$NH_2CH_2(\underline{CH_2})CH_2NH$
2.24	sep	valine	β-CH_2
2.35	m	3-D-hydroxybutyrate	α-CH_2
2.36	m	proline	β-CH_2
2.36	s	pyruvate	CH_2
2.37	m	glutamine	β-CH_2
2.40	m	glutamate*	β-CH_2
2.47	t	2-oxoglutarate	CH_2
2.51	m	3-hydroxybutyrate	CH_2
2.52†	d	citrate	CH_2
2.54	s	methylamine	CH_3
2.64	t	methionine	CH_2
2.68	dd	aspartate	CH_2
2.69†	d	citrate	CH_2
2.70	s	DMA	CH_3
2.84	dd	asparagine	CH_2
2.86	dd	aspartate	CH_2
2.97	dd	asparagine	CH_2
3.01	t	lysine	ε-CH_2
3.04	s	creatine	CH_3
3.06	dd	tyrosine	CH_2
3.12	dd	histidine	CH_2
3.12	dd	phenylalanine	CH_2
3.12	m	spermine	$NHCH_2(CH_2)_2CH_2NH$
3.13	t	spermine	$NH_2\overline{CH_2}(CH_2)\overline{CH_2}NH$
3.14	t	spermine	$NH_2\underline{CH_2}CH_2C\overline{H_2}NH$
3.16	dd	tyrosine	CH_2
3.21	s	choline	$N^+(CH_3)_3$
3.22	dd	histidine	CH_2
3.22	s	TMAO‡	$N(CH_3)_3$
3.23	s	GPC§	$N^+(CH_3)_3$
3.24	t	arginine	γ-CH_2
3.24	dd	β-glucose	H2
3.25	t	taurine	CH_2NH
3.26	dd	phenylalanine	β-CH_2
3.28	t	inositol	H5
3.36	m	proline	δ-CH_2
3.40	m	β-glucose	H4
3.41	t	taurine	CH_2SO_3

Table 1
Continued

Shift (δ)	Multiplicity	Compound	Assignment
3.42	dd	α-glucose	H4
3.45	m	proline	δ-CH_2
3.47	ddd	β-glucose	H5
3.48	dd	threonine	α-CH
3.48	m	β-glucose	H3
3.49	t	choline	NCH_2
3.53	dd	inositol	H1, H3
3.54	dd	α-glucose	H2
3.54	s	glycine	CH_2
3.57	d	valine	α-CH
3.62	dd	GPC§	NCH_2
3.66	q	ethanol	CH_2
3.63	dd	inositol	H4, H6
3.69	dd	leucine	α-CH
3.71	m	α-glucose	H3
3.72	dd	β-glucose	½ CH_2 C6
3.75	dd	isoleucine	α-CH
3.75	dd	lysine	α-CH
3.75	dd	arginine	α-CH
3.75	m	asparagine	α-CH
3.76	m	α-glucose	½ CH_2 C6
3.77	t	glutamine	α-CH
3.78	q	alanine	α-CH
3.79	dd	glutamate*	α-CH
3.84	m	α-glucose	½ CH_2C6
3.84	dd	methionine	α-CH
3.90	dd	β-glucose	½ CH_2C6
3.90	dd	aspartate	α-CH
3.93	s	creatine	CH_2
3.94	dd	tyrosine	α-CH
3.96	dd	histidine	α-CH
3.97	dd	phenylalanine	α-CH
4.02	t	inositol	H2
4.06	dd	tryptophan	CH
4.11	q	lactate	CH
4.12	m	proline	α-CH
4.13	m	3-D-hydroxybutyrate	α-CH
4.17	t	inositol	β-CH
4.20	m	GPC§	NCH_2
4.24	m	threonine	β-CH
5.80	d	uridine	cyclic H5
5.81	d	uridine	ribose H1
6.53	s	fumarate	CH=CH
6.90	m	tyrosine	H3, H5
6.99	s	histidine	H4
7.20	m	tyrosine	H2, H6
7.21	t	tyrptophan	cyclic H5/H6
7.29	t	tryptophan	cyclic H5/H6
7.33	s	tryptophan	cyclic H2
7.33	m	phenylalanine	H2, H6
7.38	m	phenylalanine	H4
7.43	m	phenylalanine	H3, H5
7.55	d	tryptophan	cyclic H7
7.71	s	histidine	H2
7.74	d	tryptophan	cyclic H4
7.84	d	uridine	cyclic H6
8.46	s	formate	CH

s, singlet; d, doublet; t, triplet; q, quartet; qu, quintet; sep, septet; m, second order multiplet; dd, doublet of doublets; ddd, doublet of doublet of doublets.

*Glutamate has anomalous chemical shifts in SF possibly due to the formation of a stable carbamic acid adduct.

†Citrate chemical shifts are highly dependent on pH and metal ion concentration. All ^{1}H chemical shifts are referenced to H1 of α-glucose at δ 5.233.

‡TMAO, trimethylamine-N-oxide.

§GPC, glycerophosphorylcholine (unstable in SF due to esterase activity but stable in SVF).

of organic disease and disturbed metabolism. In this study, having assigned in detail the ^{1}H-NMR-generated metabolic profiles of seminal, prostatic and SVFs, an example of ^{1}H-NMR spectroscopy as a diagnostic probe of perturbed reproductive tract function is now given.

A typical single-pulse 600 MHz ^{1}H-NMR spectrum with extensive signal assignment of human seminal fluid from a patient with non-obstructive azoospermia (where the infertility or lack of spermatozoa may be due to immunological problems) is shown in Fig. 9 and is compared to that from a patient with obstructive azoospermia (obstruction at the level of the ejaculatory ducts hence the ejaculate is primarily PF). Immediate gross differences in NMR-detected metabolite patterns are apparent. In particular there are many fewer resonances detectable in the tract-obstructed patient's secretions (which was typical of the 13 examined in this study) corresponding with the lack of seminal vesicle secretion. The spectrum obtained from the patients with vasal aplasia is virtually identical to that from the expressed prostatic secretions of normal patients. In obstruction, the SF spectrum is much less complex as fewer resonances were observed in the prostatic secretions. The major resonances were assigned to citrate, spermine, *myo-*

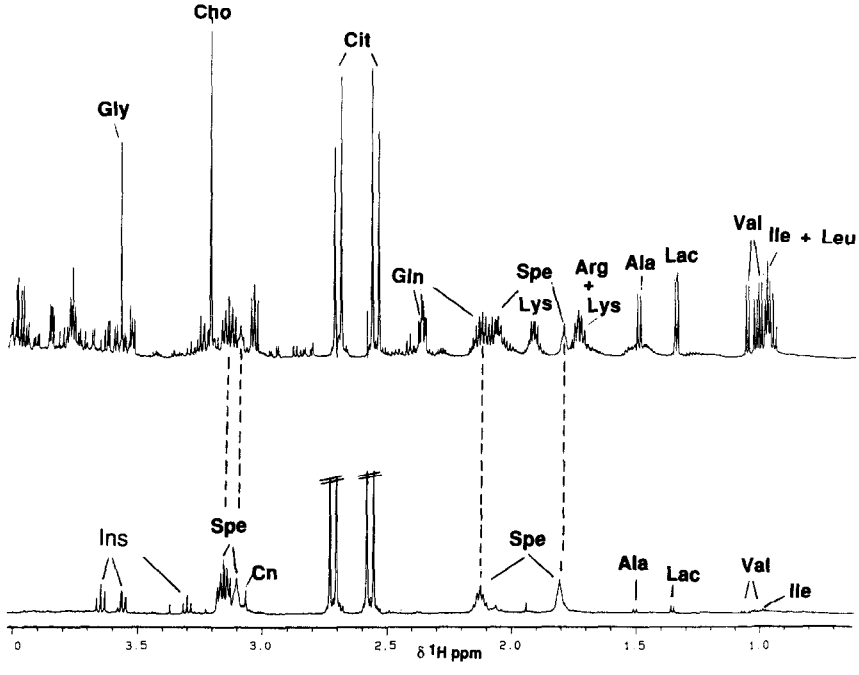

Figure 9
Single-pulse 600 MHz ^{1}H-NMR spectra of human seminal fluid from a patient with vasal aplasia (lower spectrum) and from an infertile patient with sperm antibodies but with otherwise normal prostatic and seminal vesicle function (upper spectrum).

Table 2
Relative molar concentrations of choline to citrate and spermine to choline in the seminal fluids from normal subjects, patients with vasal aplasia, and infertile patients with sperm antibodies. Values are derived from measurements of the single pulse 500 MHz ^{1}H-NMR spectra of seminal fluids. The intensities of the choline N^+-$(CH_3)_3$ singlet, at 3.21 ppm the citrate CH_2 doublet centred at 2.52 ppm, and the spermine quintet centered at 1.78 ppm were measured and relative molar concentration ratios calculated for each. Values are means ±1 standard error of the mean. *Indicates statistically significantly different ($P < 0.05$, Mann–Whitney test) from control or non-obstructed azoospermic subjects; only six of the vasal aplasia patients had peak height-derived quantitations performed because of citrate signal broadening due to metal complexation

Condition	Citrate–choline ratio	Spermine–choline ratio
Normals (n = 3)	7.92 ± 4.0	1.08 ± 0.74
Infertile (sperm antibodies) (n = 3)	11.50 ± 5.2	1.37 ± 0.9
Vasal aplasia (n = 13)	1746 ± 1107*	266 ± 136*

inositol together with minor contributions from lactate, alanine, creatine, acetate and valine. Most of the amino acid signals are absent because they are derived from prostatic peptidase activity on the seminal vesicle peptides. Furthermore, there is little or no GPC or choline as these are directly and indirectly derived from the seminal vesicle component. The expanded and integrated 500 MHz ^{1}H-NMR spectra of seminal fluids were used to calculate the molar citrate to choline, and molar spermine to choline ratios in normal, azoospermic and vasal aplasia subjects (Table 2). Very significant differences occur between subject classes with both ratios being two orders of magnitude greater for patients with vasal aplasia than in non-obstructed patients or normals. This clear finding suggests that measurement of these ratios could form the basis of a new clinical procedure for the detection of vasal aplasia or ejaculatory duct obstruction and hence the differential diagnosis of vasal aplasia, which normally requires minor surgery and/or ultrasound examination. A clinical evaluation of the use of high resolution ^{1}H-NMR spectroscopy for the detection of vasal aplasia and other disorders of the male reproductive system (including prostatic hyperplasia and cancer) is currently being undertaken together with further studies on the dynamic biochemical interactions and reactions of metabolites in seminal fluids.

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