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Journal of Pharmaceutical and Biomedical Analysis
30 (2002) 1297–1310

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Optimisation of collection, storage and preparation of rat plasma for ^{1}H NMR spectroscopic analysis in toxicology studies to determine inherent variation in biochemical profiles

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Received 24 April 2002; received in revised form 23 July 2002; accepted 30 July 2002

Abstract

Biofluid ^{1}H NMR spectroscopy has been assessed as a tool for toxicological investigations for almost two decades, with most studies focussing on urinary changes. This study has examined variations in the ^{1}H NMR spectroscopy spectra of plasma collected from control rats at different times of the day. The collection, preparation and storage of samples were optimised and potential sources of variation in samples taken for toxicology studies identified. Plasma samples were collected into heparinised containers and analysed following a standard dilution with D_2O. The value of deproteinising plasma with acetonitrile to look at low molecular weight metabolites has also been assessed. Variations in lactate and citrate levels in whole blood plasma were found and are consistent with the observation that lactate is one of the most variable metabolites in human plasma. Lipids levels also varied, in particular higher levels of lipids were found in spectra from male rats compared to female rats, and in samples collected in the morning following the feeding period. No significant changes were identified in samples which were snap-frozen and stored for up to 9 months at −80 °C. More changes were observed after storage at 4 °C or room temperature, including an increase in glycerol and choline levels, which may have resulted from lipid hydrolysis.

Keywords: Biofluid NMR spectroscopy; Rat plasma; methodology; storage; inherent variations

1. Introduction

Biofluid NMR spectroscopy has been assessed as a tool for toxicology investigations for almost two decades. Many publications have focussed on the use of ^{1}H NMR spectroscopy as a method for identifying changes in the endogenous urinary metabolites which may be indicative of toxicity or related to specific mechanisms of toxicity [1,2]. The rat is the rodent species of choice in most toxicology studies, and as a consequence ^{1}H NMR spectroscopy profiles from rat urine have been extensively described [3]. Urinary NMR spectroscopy profiles have been used in metabonomic

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0731-7085/02/$ - see front matter
PII: S0731-7085(02)00455-7

investigations to identify changes in the metabolic status of an animal over time in response to toxic events [2,4,5].

The aim of the present work was to examine changes in blood plasma levels of endogenous metabolites in control rats, and to use this methodology data in subsequent studies where plasma changes in response to xenobiotic treatment were to be investigated. Blood plasma is a complex mixture of low and high molecular weight metabolites including proteins (mainly albumin, immunoglobulins, glycoproteins and lipoproteins), together with inorganic solutes [3]. Early studies have provided comprehensive information regarding the assignment of the ^{1}H NMR spectra of plasma and the effect of different ^{1}H NMR experiments on plasma profiles [3,6–8]. Some studies also focussed on specific components of plasma. For instance, the separation, identification and quantification of lipoprotein fractions in human plasma/serum using NMR spectroscopy have recently been reported [9,10].

A number of applications of ^{1}H NMR spectroscopy of plasma in the toxicology area have also been reported: nephrotoxicity [11–13], hepatotoxicity [14,15], inborn errors of metabolism [16], inflammatory diseases [17], cancer [9,18,19], rheumatoid arthritis [20], diabetis mellitus [21]. To date, there are few metabonomic investigations of plasma where detailed pattern recognition has been carried out. Nicholls et al. have identified markers of hydrazine toxicity in plasma following its administration to rats [4]. The full potential of metabonomic investigations using plasma in combination with urine has not yet been demonstrated.

The present investigations have optimised the collection and handling of plasma samples for ^{1}H NMR analysis. The aim is to provide data on plasma to compliment urinary data and give information on the biochemical status of an animal at any stage of a preclinical investigation. Any changes identified in plasma endogenous metabolite levels as a consequence of xenobiotic treatment could correlate with urinary changes in the same animals. It is also possible that biochemical changes could be detected at an earlier time point in plasma.

Before using plasma for metabonomic investigations, contitions were optimised for blood collection and plasma preparation. Potential sources of inherent variation were also identified. Plasma was prepared and analysed by ^{1}H NMR spectroscopy from control male and female rats at different times of the day to determine inter-animal variation and variation due to gender, diet and/or the diurnal cycle. The Wistar Han rat was chosen as it was the strain currently used in toxicology studies.

Different methods of plasma sample preparation were compared to determine the most appropriate as a routine screening method for plasma, and, which would enable identification of small metabolites. Precipitation of plasma proteins reduces the interactions that exist between proteins and low molecular weight metabolites. This provides additional biochemical information by rendering 'NMR visible' some formerly 'NMR invisible' molecular species [3,22]. Various methods of sample preparation have been used for plasma in published work, but only recently has a comparison of some of the methods to deproteinise plasma been published to our knowledge [23]. This comparative work was developed on human plasma, not rat plasma [18,24–26]. Ultrafiltration, solid phase extraction chromatography and precipitation with organic solvents were compared. The precipitation of proteins in plasma with acetonitrile (ACN) at physiological pH proved to be a useful method for the quick and easy release of many small molecules. An in-house method for the precipitation of proteins in rat plasma using ACN was therefore developed. The effect of storage conditions was also studied to assess the stability of rat plasma for up to 9 months.

2. Experimental

2.1. Chemicals

Choline, dimethylamine hydrochloride (DMA), D-fucose, D-(+)-glucose, L-glutamic acid, L-glutamine, glycerol, L-histidine, L-proline, taurine, L-threonine, L-tyrosine, trimethylamine oxide (TMAO) and acetonitrile (ACN) were purchased from Sigma-Aldrich (Gillingham, Dorset, UK).

2.2. Animals and treatments

Six male and three female Wistar Han rats (Charles River laboratories) aged 15–16 weeks were housed in polycarbonate cages (Techniplast UK) in a room with regular light cycles (12 h). Food (rat and mouse No.1 Expanded Diet, SDS, UK) and tap water were provided ad libitum. Blood was collected at autopsy under isoflurane anaesthesia by abdominal exsanguination (approximately 8 ml of blood per animal) into 10 ml-lithium-heparin or K_2 EDTA containers. Animals were divided into three groups of three rats each. A first group (1) was composed of males (average weight: 380 g) from which blood was collected between 08:00 and 10:00 h. A second group (2) was composed of females (average weight: 224 g) from which blood was also collected between 08:00 and 10:00 h. A third group (3) was composed of males (average weight: 384 g) from which blood was collected between 16:00 and 18:00 h.

2.3. Preparation and storage of plasma samples

2.3.1. Whole plasma

Tubes containing blood were immediately placed on a roller for up to 35 min prior to centrifugation for 15 min (approx. $1500 \times g$, 20 °C). Plasma was divided into 0.5 ml aliquots in 1.5 ml Sarstedt™ tubes and either analysed immediately or stored at −20 °C or −80 °C. Some blood samples were collected into containers with no-anticoagulant and were left to clot at room temperature for 1 h prior to centrifugation in order to prepare serum.

D_2O or sodium chloride in D_2O (1.6% w/v, final concentration 0.45% w/v) was added to aliquots (approx. 500 μl) of fresh or snap-frozen plasma samples (defrosted at room temperature), to give a 40% dilution. Following centrifugation (20 min, $2400 \times g$, 4 °C), supernatants were transferred into 5 mm Wilmad 507 PP NMR tubes. The tubes were inverted several times to ensure thorough mixing. A further 10% dilution of some samples was carried out with a sodium azide (1% (w/v) in D_2O to assess the need for an antibacterial agent. Plasma samples diluted at 100% (300 μl plasma + 300 μl D_2O) and 400% (100 μl plasma + 400 μl D_2O) were also prepared to assess the effect of dilution.

2.3.2. Deproteinised plasma

Aliquots of whole plasma (approx. 500 μl) stored at −80 °C were defrosted at room temperature. The volume (V_{pl}) of plasma was measured and an equivalent volume of ACN added prior to vigorous mixing. The sample was allowed to stand for approximately 5 min to allow proteins to precipitate, then centrifuged (5 min, $13\,500 \times g$, room temp.). Solvent was removed from the supernatant under a stream of nitrogen (30–60 min) then freeze-dried for 15–20 h, or 5–6 h when stated. The residue was reconstituted with D_2O to a final volume equivalent to a 40% dilution of the initial volume of plasma V_{pl} and transferred into NMR tubes. A range of sample preparation methods were used which included dilution with phosphate buffer (pH 7.4, 0.1 M) and analysis without solvent removal to examine any metabolite loss during solvent removal. A 10% dilution with D_2O was made (final volume of approximately 1.1 ml) before analysis.

2.3.3. Addition of standards

Solutions of standards (refer to the chemical list) were added to plasma samples (500 μl of whole or deproteinised plasma) as 10 μl aliquots of 5 or 50 mM aqueous solutions, giving 0.1 or 1.0 mM final concentration. Sodium hydroxide was added to standard solutions to achieve complete dissolution of the substance if necessary.

2.3.4. ^1H NMR analysis

^1H chemical shifts were referenced internally to the α-glucose H1 resonance at δ 5.236, previously measured relative to the primary internal chemical shift reference trimethylsilyl [2,2,3,3-$^2H_4$]propionate (TSP) at δ 0.00. Samples were analysed at 600.13 MHz on a Bruker DRX-600 spectrometer at ambient probe temperature (±300 K) using a 5 mm TXI ^1H probe. In order to suppress the large water signal, spectra were acquired with the pulse sequence NOESYPR1D:

$$RD - 90° - t_1 - 90° - t_m - 90° - \text{collect FID}$$

where RD is a relaxation delay of 2 s, during which the water is selectively irradiated; t_1 represents the first increment in a NOESY experiment and is set to 3 μs; and t_m had a value of 100 ms, during which the water resonance was again selectively irradiated. One hundred and twenty eight scans and four dummy scans were collected into 64k computer data points, with a spectral width of 12 019.23 Hz, an acquisition time of 2.73 s and a relaxation delay of 2.00 s (total recycle delay of 4.73 s). Spectra were also acquired using a Carr–Purcell–Meiboom–Gill (CPMG) spin-echo experiment (with NOESYPR pre-saturation of the water resonance) using the following pulse sequence:

$$RD - 90^\circ_x(\tau - 180^\circ_y - \tau)_n - \text{collect FID}$$

with 128 scans (n) and four dummy scans into 64k computer data points, a spectral width of 12 019.23 Hz, an acquisition time of 2.73 s and a relaxation delay of 2.00 s (total delay between pulse cycles of 4.73 s). The fixed delay τ, set at 830 μs, allows spectral editing via faster T_2 relaxation and hence attenuation of broad signals. The total spin–spin relaxation delay ($2n\tau$) was 212.5 ms. Spectra of samples containing both water and ACN were acquired with a dual solvent suppression sequence.

The free induction decays from each sample were multiplied by an exponential line broadening function of 0.30–0.50 Hz prior to Fourier transformation, for all experiments. Where stated, the spectral intensities were normalised by adjusting them arithmetically, to take into consideration both the number of scans and receiver gain, using a utility program (Normpor) written by Dr A. Gibbs, Bruker Biospin, Banner lane, Coventry, UK.

At least three separate plasma samples were analysed for each methodology.

3. Results and discussion

3.1. Development of a methodology to optimise acquisition of ^1H NMR spectra of plasma samples

Plasma is routinely prepared in toxicology studies, which makes this (rather than serum) the biofluid of choice for analysis. In addition, ^1H NMR spectra of plasma and serum looked similar, therefore, plasma was selected for all further work.

The concentration changes reported here were calculated by normalising each metabolite relative to the total metabolite content of each sample. This is a standard method of normalisation, often used in many branches of data analyses, and particularly spectroscopy, but has the limitation that only relative, or semi-quantitative changes can be obtained. One approach to obtaining absolute quantitation for each component would employ the use of an appropriate internal standard, which is not found endogenously and is not protein bound. An investigation is currently in progress to search for a suitable internal standard and will be reported separately.

3.1.1. Blood sampling and NMR experiments

The use of microtainers™ (Becton Dickinson, USA) to collect blood was avoided as it generated interfering signals in the ^1H NMR spectra (Stanley, personal communication). For a similar reason, containers filled with lithium-heparin as an anti-coagulant were preferred to EDTA containers. Time between blood sampling and plasma separation was found not to be critical up to 35 min, as there were no changes in small metabolites normally associated with cell leakage. In addition there was no evidence of haemolysis over the 35-min period.

Both proton NOESYPR1D and CPMG spin-echo spectra were recorded for each sample. Spin-echo methods eliminate broad lines from macromolecules such as albumin and lipoproteins, which have short transverse relaxation times T_2. Changes in lipid (mainly lipoproteins) and glycoprotein resonances were therefore more easily observed in NOESYPR1D spectra and those relating to low molecular weight molecules in CPMG spin-echo spectra [7].

Any changes in peak intensities of endogenous metabolites in NOESYPR1D and CPMG NMR spectra were estimated relative to glucose resonances (δ 4.64 and 5.24 for β- and α-glucose H1, respectively) unless otherwise stated since signals of the anomeric protons exhibited low variability relative to noise levels. However it is recognised

that this 'normalisation' method may not be accurate when plasma samples from toxin treated animals are examined. Plasma glucose levels may change as a result of kidney damage leading to an imbalance in energy intermediates, reflect a disease state, such as insulin-dependent diabetes (Diabetes mellitus), or change simply as a result of fasting. Indeed glucose levels have been shown to decrease gradually in the plasma of a normal subject who was fasted for 12, 37 and 48 h [21].

3.1.2. Overview of various methods to prepare plasma samples

^{1}H NMR spectra recorded on whole plasma provided general information on a wide range of endogenous metabolites. Samples of whole plasma were routinely diluted at 40% with deuterated water. Dilution at 40% with a saline solution made of sodium chloride in D_2O (final concentration: 0.45% w/v) was assessed to help maintain the ionic balance of plasma. However, the spectra were identical. Ammonium has been reported to be effective for releasing metabolites, such as lactate, bound to proteins in human plasma or serum [27,28]. However, resolution of peaks was not improved by the addition of ammonium chloride.

3.1.2.1. Effect of dilution with D_2O. The experiments described in the present study were carried out with 500 μl plasma and 200 μl D_2O. However, volumes of plasma collected in toxicology studies are often less than 500 μl. For instance the volume of 100 μl-samples has to be made up to 500 μl at least by addition of D_2O (400% dilution). Therefore the effect of a dilution at 100 or 400% was investigated. In all samples diluted at 400%, a high-field chemical shift of approximately 0.01 ppm of some amino-acids resonances was observed on CPMG spectra (Fig. 1). These amino-acids were mainly alanine, valine, isoleucine, histidine, tyrosine, glutamine and glycine. The binding of small molecules to proteins might be affected by the dilution and thus alter their resonances shifts and intensities. A slight change in pH and cation concentrations can also affect chemical shifts (Stanley, personal communication). As no change was observed in peak intensities, it seems likely that slight changes in pH or cation concentration are the most likely explanation for the chemical shifts. In two out of three of the 400% diluted samples, an increase in citrate levels was also observed (Fig. 1).

The efficiency of selective extraction procedures to isolate other molecules present in whole plasma, such as macromolecules (e.g. lipids) or small molecules which may be protein bound, was also assessed.

3.1.2.2. Extraction of lipids from plasma. The major lipids present in plasma are triglycerides, cholesterol and phospholipids, transported as lipoprotein complexes or free fatty acids bound to albumin [29]. It has been suggested that all plasma lipids showing signals by ^{1}H NMR are in lipoproteins including chylomicrons [21]. Initial separation of lipids for ^{1}H NMR analysis from the lipid-soluble components in 'freeze-dried' plasma was carried out using methanol extraction [30]. However, it was not selective enough as lipids such as cholesterol and small metabolites such as glucose were still found in the extracts. A more effective method to selectively extract lipids was a chloroform/methanol (2:1) extraction with several washing procedures to remove all small aqueous soluble components [19,31]. This method is time-consuming but could be applied in specific cases where the lipid components of plasma are of particular interest.

3.1.2.3. Precipitation of proteins from plasma. Some low molecular weight metabolites such as aromatic amino acids (tyrosine and phenylalanine), histidine, lactate and citrate have been shown to be bound to plasma proteins [24,27]. This results in the attenuation or even suppression of the resonances in NMR spectra due to a decrease in T_2 relaxation times. Other small metabolite signals may be overlapped by broad resonances from macromolecules. Methods for precipitating proteins have been investigated to enable more resonances from small endogenous metabolites to be seen and hence improve interpretation of the data. Preliminary work using ACN to precipitate proteins showed no differences in the spectra of plasma extracted with 1 or 4

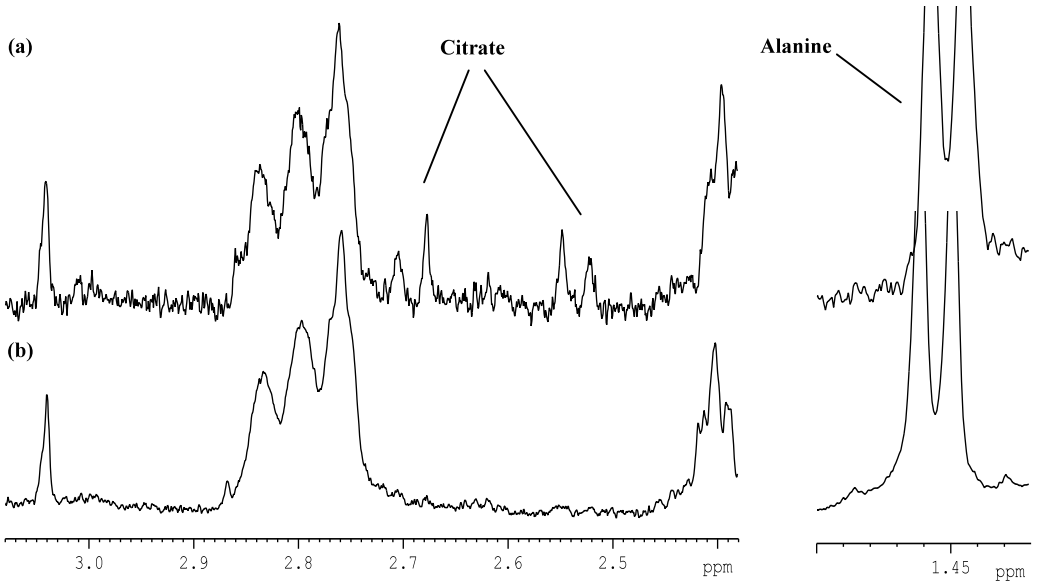

Fig. 1. ^{1}H NMR CPMG spectra of pooled control plasma from male rats (Group 3). Samples were stored at −80 °C and diluted (a) by 400% with D_2O, (b) by 100% with D_2O. Spectra were normalised so that the spectra profiles look similar.

volumes, therefore only 1 volume of solvent was added.

Some residual broad resonances were still present in NOESYPR1D spectra from the deproteinised plasma, which overlapped with some small metabolites such as 3-hydroxybutyrate at δ 1.20 and threonine at δ 1.27, making CPMG experiments more suitable for analysis.

Spectra to be compared were normalised (Normpor) to compare peak intensities before and after protein removal.

3.1.2.4. Spectral changes following extraction. There was a decrease in signal intensity of most resonances after extraction although a number of new peaks were revealed in the aromatic region between δ 6.8 and 7.8 and in the aliphatic region, due to the removal of almost all lipid broad resonances (δ 1.20–1.30, 2.00–2.05, 2.75–2.85) and N- and O-acetyl glycoprotein signals (δ 2.04, 2.08 and 2.14) (Fig. 2).

Some of the metabolites with improved signals following protein precipitation were: threonine (δ 1.27), glutamate (δ 2.36), a singlet at δ 2.08, dimethylamine (δ 2.74) and trimethylamine (δ 2.88), isoleucine (δ 0.93 and 1.00), leucine (δ 0.95, 0.97 and 1.71), 3-hydroxybutyrate (δ 1.20), and other unassigned metabolites. Lactate levels were not increased in rat plasma, contrary to previous results, which reported a high proportion of protein binding of lactate has been seen in human plasma [23,27]. However, the peak intensities of phenylalanine (δ 7.33, 7.38 and 7.43) and tyrosine (δ 6.87 and 7.17) were increased which is in agreement with the previously reported binding of these molecules in human plasma [22]. The intensities of the two doublets of tyrosine were not equivalent in whole plasma, suggesting the more intense signal was possibly assigned to another molecule as well as to tyrosine. Following ACN extraction the two doublets showed similar intensities suggesting that the molecule had been removed by the extraction process.

A slight high-field shift of 0.04 ppm was observed for tyrosine, histidine, valine and alanine resonances. It was reversed by a 20% dilution with phosphate buffer at pH 7.4. Similar shifts have been previously reported for alanine in lyophilised plasma reconstituted in D_2O and for components of urine (e.g. histidine) due to minor pH changes [30,32]. Resonances of citrate doublets in whole plasma were δ 2.52 and 2.69. However shifts to δ

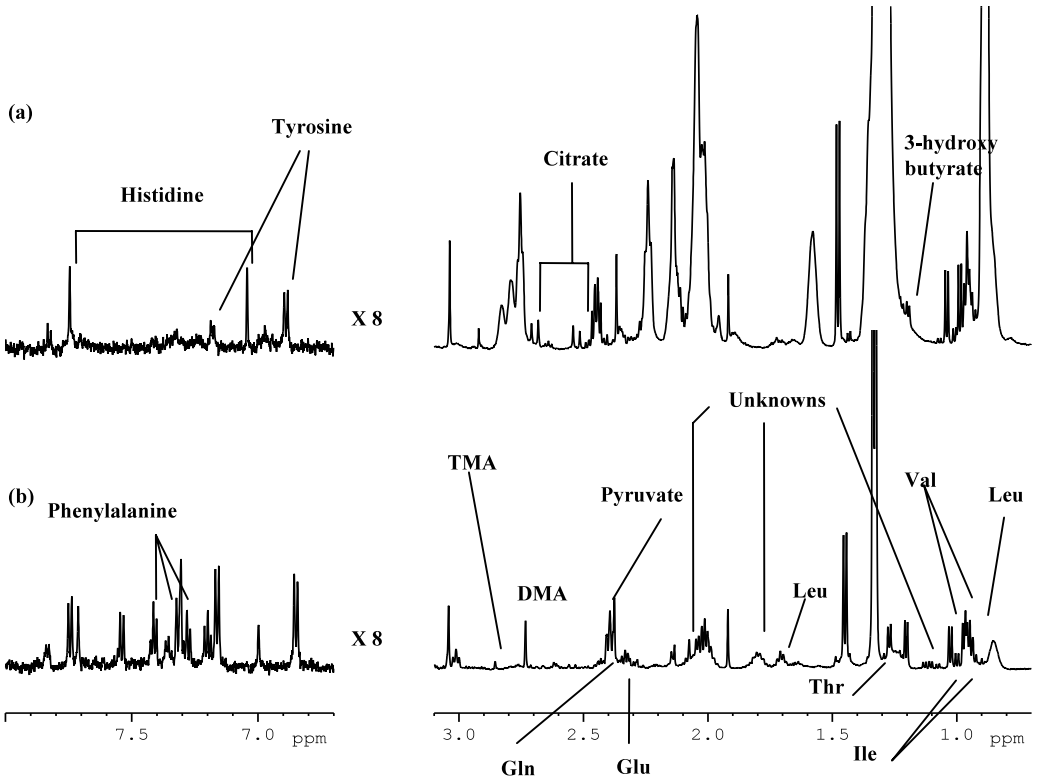

Fig. 2. ^{1}H NMR CPMG spectra of control plasma from a male rat (Group 1). Samples were stored at −80 °C. Spectra were normalised. (a) Whole plasma, (b) plasma deproteinised with ACN. The main changes in endogenous metabolites are indicated. DMA, dimethylamine; TMA, trimethylamine.

2.54 and 2.66 were observed in deproteinised plasma, even in the presence of phosphate buffer. Similar shifts have been previously reported for citrate in urine (Stanley, personal communication).

Citrate levels decreased significantly in deproteinised rat plasma although they have previously been shown to increase in human plasma after protein precipitation with ACN, which was likely to be due to its release from albumin [23]. The amount of citrate bound to albumin may differ in rat plasma and human plasma. The same group [23], also reported a loss of citrate in human plasma when ice-cold acetone was used to remove protein. This suggests that the mechanism of unfolding and/or degree of denaturation of proteins is different at room temperature and at 4 °C or that protein precipitation with acetone does not release all of bound citrate. Therefore, the denaturation conditions may have been slightly different in the method used in this study. Citrate may also have been lost during freeze-drying due to its volatility. This last hypothesis was supported by the observation that citrate had not decreased in the samples analysed prior to solvent removal and in samples which were freeze-dried for 5–6 h instead of 15–20 h.

A slight decrease in the level of formate (δ 8.47) was also attributed to its volatility and loss during freeze-drying, as levels were unchanged in deproteinised plasma analysed in samples prior to removal of ACN. Other volatile compounds with signals in the aromatic regions were also present in deproteinised plasma samples analysed before ACN was removed before analysis. However, analysis of sample extracts before ACN removal is not ideal as the samples are more dilute (final volume 1.1 ml vs. 0.7 ml for freeze-dried samples) and residual broad resonances were present in the

δ 0.8–1.0 and δ 1.3–1.5 regions. Information was complicated in the δ 2.0–2.3 region because of the suppression of the ACN signal at δ 2.13 and the appearance of ^{13}C satellites at δ 2.01 and δ 2.24 (e.g. acetoacetate at δ 2.22). Dimethylamine (DMA), trimethylamine (TMA) and some aromatic compounds were also not visible in the dilute extracts. These results suggest that it is better to freeze–dry extracts following deproteinisation with ACN.

The reproducibility of extracting samples after freeze-drying for 5–6 or 15–20 h was investigated. All CPMG spectra were normalised prior to comparison (Normpor) and showed negligible differences in levels of metabolites, except for the appearance of the unknown singlet at δ 2.08.

Some endogenous metabolites, previously described as potential biomarkers of toxicity in rat plasma, may be easier to identify in plasma deproteinised with ACN rather than in whole plasma. Examples of these metabolites were those seen in rats following treatment with hepatotoxins (e.g. arginine, Nicholls, personal communication), nephrotoxins (3-hydroxyisobutyrate, dimethylamine [33]), or in rats treated with either of these types of toxin (3-hydroxybutyrate, acetoacetate [14,33]). The NMR signals of these molecules are either overlapped or 'invisible' on spectra.

Using the extraction methods described here, a greater number of small endogenous metabolites were observed in plasma deproteinised with ACN. The loss of volatile plasma components during freeze-drying was avoided by reducing the duration of the freeze-drying process. As the method is not too time-consuming, is reproducible and provides information on small metabolites which are otherwise masked, it was recommended that both whole and deproteinised plasma samples be analysed in future studies. Addition of a suitable internal standard prior to protein removal is being investigated as this would enable the % recovery of metabolites to be assessed during the procedure.

3.1.3. Effect of storage conditions

3.1.3.1. Effect of storage at room temperature. The effect of maintaining plasma samples (diluted to 40% with D_2O) on the autosampler of the NMR spectrometer for 15 or 24 h at room temperature was investigated. Spectra from snap-frozen plasma obtained immediately after thawing were used for comparison. It appeared that plasma left for either 15 h (equivalent to an over-night run on the autosampler) or 24 h at room temperature were affected to the same extent. One detectable change was a slight increase in peak intensities of tyrosine (δ 6.87 and 7.17) and phenylalanine (δ 7.33, 7.38 and 7.43) (Fig. 3). This increase was likely to be the consequence of a loss of macromolecules (probably as a precipitate) and subsequent release of bound aromatic aminoacids. A similar change in the aromatic region was seen when plasma was deproteinised (Fig. 2b). Tyrosine could have been produced enzymatically from phenylalanine but a decrease in phenylalanine levels would have been expected over the same time period. Both may be increased due to protease activity. A second significant change was the increased signals of glycerol at δ 3.56 and 3.64 (similar to those shown on Fig. 4b).

3.1.3.2. Effect of storage at 4 °C. The effect of storing plasma for 1 week at 4 °C was investigated in plasma samples diluted to 40% with D_2O. The comparison was made between snap-frozen plasma analysed just after thawing and again after storage for 1 week at 4 °C. Spectral changes of human plasma stored at 4 °C for as long as 6 days have been reported to be negligible [26]. However, a decrease in methyl and methylene resonances of lipids in VLDL and chylomicrons and a broadening of all methyl and methylene resonances assigned to lipids has also been reported in human plasma after a prolonged storage (3–4 days) of samples at 4 °C [25]. Only a slight decrease in these resonances was seen in the NOESYPR1D spectra in our study. 3-Hydroxybutyrate (δ 1.20), acetate (δ 1.91) and glycerol (δ 3.56 and 3.64) levels slightly increased while pyruvate levels (δ 2.36) decreased (Fig. 4).

The unknown singlet at δ 2.08 seen in extracted samples, was slightly increased but remained at very low levels compared with levels observed in fresh plasma. This singlet cannot be assigned to a contaminate as it was not seen in the spectra of the same plasma samples analysed before storage at

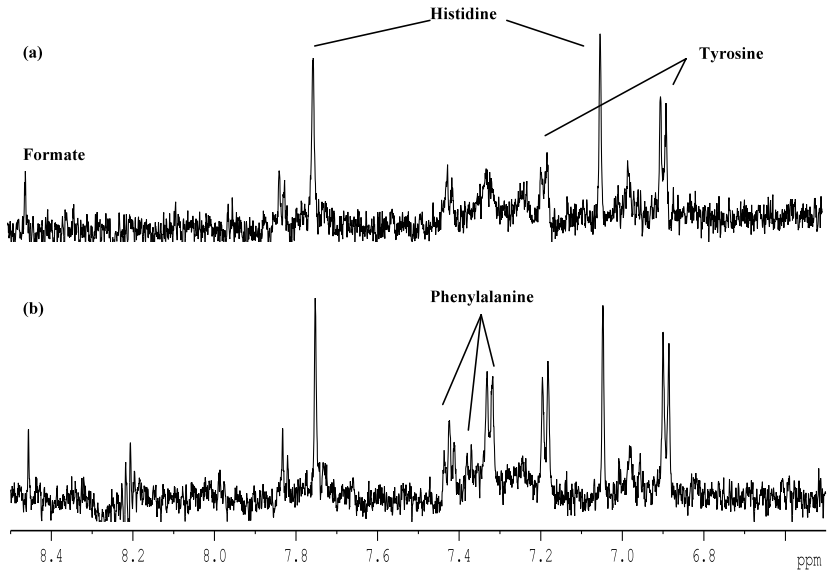

Fig. 3. ^{1}H NMR CPMG spectra of control plasma from a male rat (Group 3). (a) Snap-frozen plasma stored 48 h at −80 °C, (b) same sample after 24 h at room temperature.

4 °C (samples were retained in the same tubes until analysis 1 week later). Another increased singlet at δ 3.20 was assigned to methyl groups in choline; this was confirmed by addition of a standard solution of choline. Choline may have been released from phospholipids, such as phosphatidylcholine, contained in lipoprotein membranes. However, enzymatic lysis can also produce phosphocholine (lecithin) instead of choline which would have the same chemical shift at δ

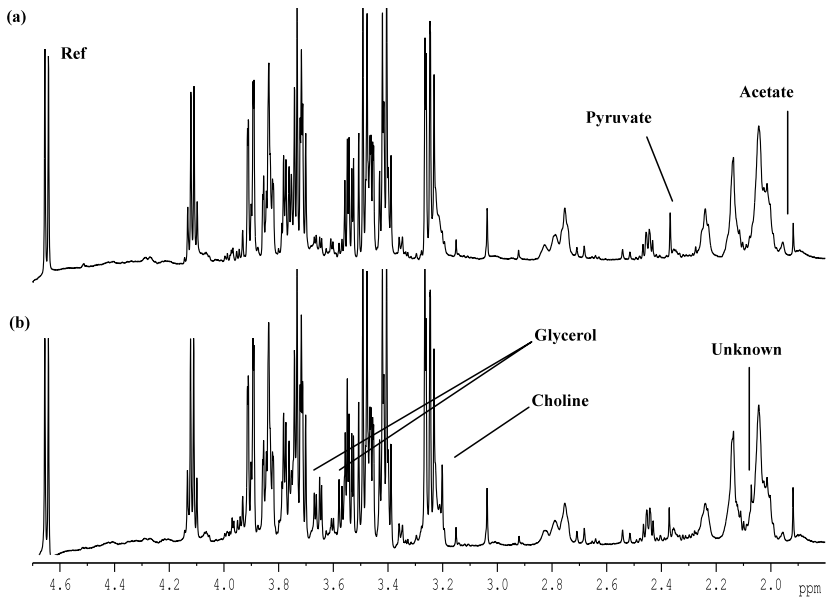

Fig. 4. ^{1}H NMR CPMG spectra of control plasma from a male rat (Group 1). Samples were snap-frozen and stored 48 h at −80 °C prior to NMR analysis (a) on day of thawing or (b) after a 1-week storage at 4 °C.

3.20 [29]. In a similar manner, the observed increase in glycerol may have resulted from the hydrolysis of some lipids by action of lipases. Triglycerides in plasma are removed from chylomicrons and VLDL by the action of lipoprotein lipase (LPL) [29]. This results in the formation of glycerol and free fatty acids, which are subsequently delivered to adipose or muscle tissues. The suggestion that enzymatic activity may be responsible for some of the changes seen in the samples stored for up to a week at 4 °C, is supported by the fact that none of these changes were observed in deproteinised plasma stored for 3 weeks at 4 °C.

Similar increases in tyrosine and phenylalanine levels to those observed in plasma stored 24 h at room temperature were seen in the aromatic region. This is in agreement with a previously reported gradual increase of all amino-acid concentrations in rat plasma stored at 4 °C [34] and may be due to protease activity.

3.1.4. Effect of snap-freezing and thawing

^{1}H NMR spectra from plasma samples which had been snap-frozen and stored for 48 h at −80 °C were compared with spectra from fresh plasma samples. A slight decrease in some lipids resonances at δ 0.8–0.9 (methyl region), δ 1.2–1.3 and 1.57 (methylene region) and δ 5.3–5.4 (methine region) was observed in spectra from snap-frozen plasma from one animal only. The broadening of lipoprotein resonances has previously been described in human plasma stored at −20 °C for 5 days [24,25]. This broadening increased with the number of freezing/thawing cycles and was attributed to a decrease in chylomicrons and VLDL sharp resonances, probably as a result of protein precipitation following the freezing/thaw process. In this study, a decrease in plasma citrate levels (δ 2.52 and 2.69) was observed in one animal, as well as the loss of an unknown peak at δ 2.08 which was present in two of the three fresh plasma samples when they were first analysed.

3.1.4.1. Effect of storage at −20 or −80 °C. The effect of a short- and long-term storage of frozen plasma was assessed. The effect of storage at −20 or −80 °C for 1 month was first investigated and no difference was found. This observation is similar to previously reported results following storage of control human plasma for up to 10 days at −20 °C [18]. Our results suggest that the storage of whole rat plasma at −20 or −80 °C results in similar spectra. These results are different from those reported in a study to investigate the influence of storage conditions on the amino-acid concentrations in rat and human plasma where changes in some amino-acid concentrations observed in rat plasma stored at −20 °C but not at −70 °C [34]. The authors suggested that freezing may not inhibit all enzymatic activity and that the degree of inhibition might vary from species to species. Therefore, plasma from different species may require different storage or processing techniques.

The effect of storage at −80 °C for up to 9 months has also been assessed. A plasma sample was divided into a number of separate aliquots which were frozen at −80 °C. Three samples were thawed and analysed after 48 h, 3 and 6 months. No differences were seen in these nine samples. A further nine samples were analysed after 9 months. The only change observed in two out of these nine samples was a slight high-field chemical shift (0.01–0.02 ppm) of some amino-acids resonances and the disappearance of citrate resonances. Interestingly, a similar decrease in citrate resonances was also seen in one out of 3 plasma samples as a consequence of thawing. A change in the protein composition of the samples may result from long-term storage or thawing. This would modify interactions between macromolecules and small molecules such as amino acids, and hence affect their NMR signal. The observed chemical shift may also be due to a change in pH.

Overall, storage at −80 °C for the first 6 months does not seem to affect the composition of plasma to any significant extent.

3.1.4.2. Effect of sodium azide addition to samples. The addition of a solution of the bactericide sodium azide did not prevent the appearance of any of the changes reported following different storage conditions, demonstrating that the

changes were not the result of bacterial degradation.

3.2. Inherent variations in plasma samples

Although all rats were the same age, differences in their body weights and activity may explain some of the variability seen between plasma samples from the same strain of rats. It is also likely that factors such as dietary intake and the period of time since food was last ingested explains other aspects of the variability observed between individuals.

3.2.1. Inter-animal variability

^{1}H-NMR spectra of fresh plasma prepared from male rats (group 1) were compared.

Most of the differences between the samples were in the regions corresponding to lipids resonances: δ 0.8–0.9, δ 1.2–2.9, δ 5.3–5.4) (Fig. 5). Notable differences in levels of lipids contained in VLDL were observed in the methyl region where they contribute to the sharpening of the signal. Variable signals were also observed in the region between the N-acetyl and O-acetyl groups of glycoproteins at δ 2.04 and 2.08, and δ 2.14, respectively. The sharp and dominant unknown singlet at δ 2.08 was seen in two of the three plasma profiles. There were also marked differences in lactate (δ 1.33 and 4.11) and citrate (δ 2.52 and 2.69) levels. Lactate has been described as one of the most variable metabolites in human plasma, together with other small metabolites such as acetate, citrate, methylamine and TMAO [7].

No differences were observed in the aromatic proton region.

^{1}H-NMR spectra of plasma from female rats (group 2) were also compared. Similar differences between samples were seen in these samples as the male samples. In addition, levels of TMAO (δ 3.26) also varied in untreated plasma from females. The assignment of the peak at δ 3.26 was attributed to TMAO, rather than to betaine, as the singlet at δ 3.92 was not present. However, this was not confirmed by a 2D-experiment.

3.2.2. Inter-gender variability

^{1}H-NMR spectra of plasma samples from males and females in groups 1 and 2, were compared. All six samples were snap-frozen and stored for 48 h

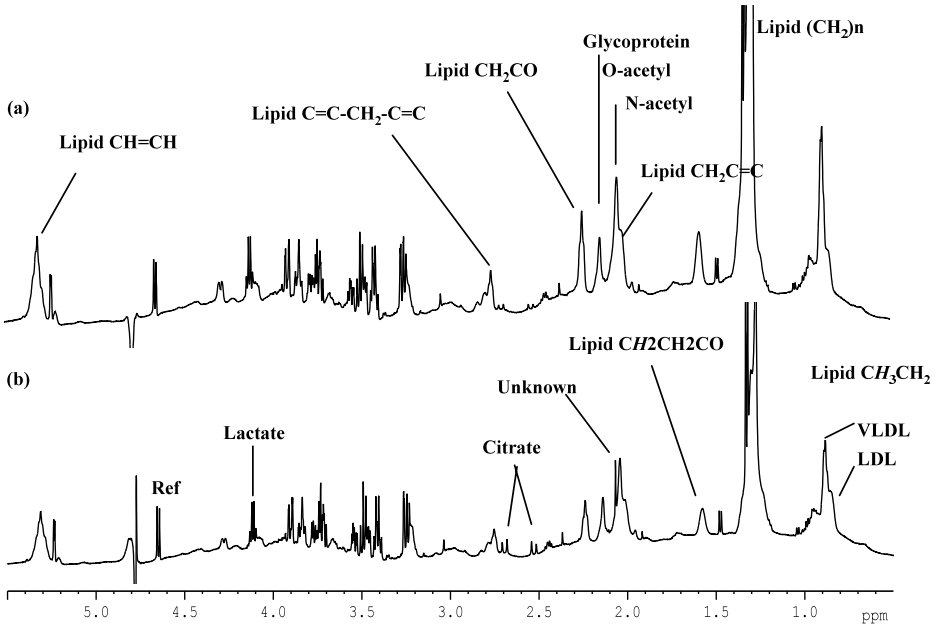

Fig. 5. ^{1}H NMR NOESYPR1D spectra of fresh control plasma from two male rats (Group 1). The main variations in endogenous metabolites are indicated. (V)LDL, (very) low density lipoproteins.

prior to analysis. Differences were observed in the regions of the spectra corresponding to lipid resonances (δ 0.8–0.9, δ 1.2–2.9, δ 5.3–5.4) (Fig. 6). Some variations in similar regions have already been described in this report, but higher levels of lipids were usually found in spectra from male rats. In particular, VLDL lipid levels at δ 0.87 and 1.29. These differences in lipid levels may be explained by the considerable body weight difference between male and female rats of a similar age. The average weights of males in groups 1 and 3 were respectively 380 g and 384 g, whereas the average weight of the female rats in group 2 was only 224 g. No difference in the aromatic proton region was observed. The effect of body weight on plasma profiles is under investigation.

3.2.3. Differences between morning and evening plasma collections

Previous studies have compared plasma from human volunteers following a meal and after an overnight fast. It was concluded that there were sharper resonances in the lipid regions of the spectra following food intake [24,25]. These resonances were attributed to an increase in chylomicrons (the major transport form of dietary fats in plasma) and VLDL in plasma.

In the present study, ^{1}H-NMR spectra of plasma from male rats in groups 1 (08:00–10:00 h collection) and 3 (16:00–18:00 h collection) were compared. All six samples were snap-frozen and stored for 48 h prior to analysis. Lipid resonances (δ 0.8–0.9, δ 1.2–1.3, δ 1.57) were lower in spectra from samples collected in the evening. These data are therefore consistent with the nocturnal feeding habits of these animals. Creatine (δ 3.04), pyruvate (δ 2.36), TMAO (δ 3.26) and lactate (δ 1.33 and 4.11) levels were also lower in samples collected in the evening. Even though changes in lactate levels have been observed between individuals in the present study, the changes described here were observed in all three samples collected in the evening. No difference in the aromatic proton region was observed in samples taken in the morning and evening.

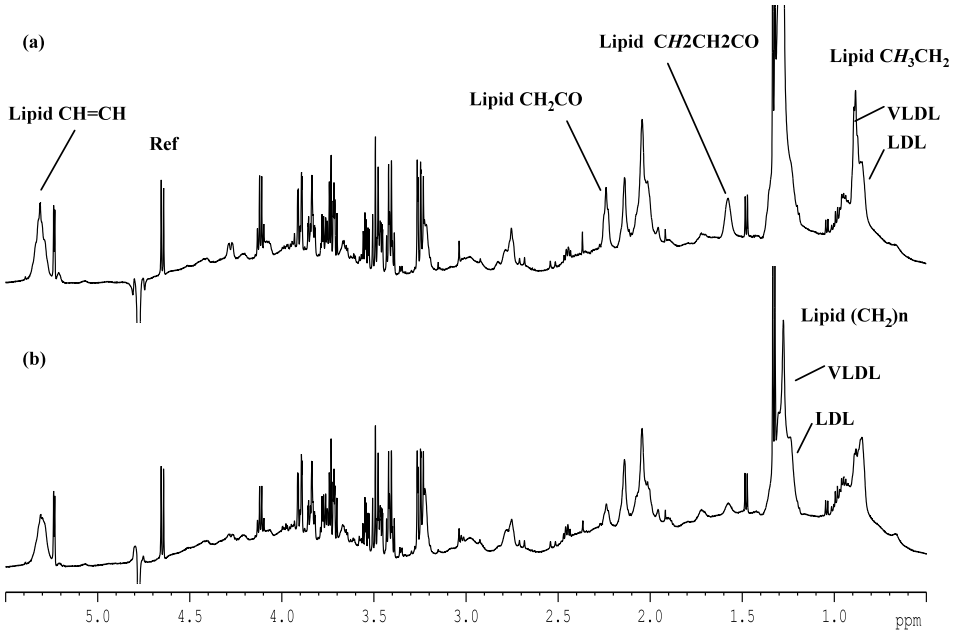

Fig. 6. ^{1}H NMR NOESYPR1D spectra of control rat plasma stored 48 h at −80 °C. (a) Male, Group 1; (b) female, group 2. The main changes in endogenous metabolites are indicated.

4. Conclusions

A methodology for the analysis of rat plasma by ^{1}H NMR has been established. Blood was preferentially collected into heparinised containers and all resulting plasma samples diluted 2-fold with D_2O. ^{1}H NMR analysis of plasma in toxicology studies could include the analysis of (i) whole plasma (with both NOESYPR1D and CPMG experiments) to provide an overview of plasma components and (ii) plasma deproteinised with ACN to provide more complete information about small metabolites. The full potential of the latter method, including quantitation of metabolites, is being investigated further by incorporating an internal standard. In some specific cases methanol/chloroform extracts could be prepared to look at lipid profiles.

No important changes were related to 'snap-freezing' and thawing, even after storage at −80 °C for 1, 3 or 6 months. Storage for 9 months resulted in a few changes in two out of nine samples, while there was no difference between samples stored for 1 month at −20 °C and −80 °C. More significant changes were observed after storage at 4 °C or room temperature, including a notable increase in glycerol and choline which was probably due to enzymatic activity in the samples.

The present work has also provided background information on endogenous metabolite levels observed in control plasma from Wistar Han rats. The most marked differences were seen in the levels of lipids, which show inherent variation particularly in the evening plasma collections and in female rat plasma, where the sharp resonances of VLDL (and chylomicrons) were low. Lactate, citrate and TMAO were also among the most variable metabolites in plasma.

Some recommendations can be made which take these findings into account.

4.1. Recommendations for sample preparation

1) As no difference was observed in small metabolite profiles of plasma and serum, it is suggested that plasma can be used for routine analysis.
2) Heparin is the recommended anticoagulant as EDTA gives additional signals in ^{1}H-NMR experiments. Microtainers^{TM} should not be used to separate blood cells from the plasma as this also resulted in additional signals.
3) As no difference was seen in the small metabolite profiles of plasma from blood samples left to mix with anti-coagulant for up to 35 min, a sample "rolling time" of up to 35 min is recommended.
4) To identify larger molecules in the sample, such as lipids, as well as small metabolites, plasma samples could be divided into two aliquots. The first should be analysed following simple dilution with D_2O (with a constant factor of dilution—ideally, 300 μl plasma diluted 2-fold), and the second treated by an appropriate extraction method to identify specific metabolites of interest.

4.2. Recommendations for plasma storage

1) Significant changes in the concentrations of some metabolites were seen after storage at 4 °C or room temperature. These included increases in choline and glycerol resonances, which were probably the result of lipase activity. Unless a lipase inhibitor is added to whole plasma to prevent lipolysis, it is recommended that plasma samples be analysed as soon as they are defrosted. The samples should not be kept for more than 8 h on the NMR spectrometer autosampler.
2) Storage of whole rat plasma for 1 month at either −20 or −80 °C resulted in identical spectra. However, as some metabolites are present in very low concentrations in plasma they were not observed in NMR spectra but they may also be subject to changes during storage. By default, it is recommended to snap-freeze and store plasma samples at −80 °C.

4.3. Spectral differences due to physiological variation

VLDL and chylomicrons were reduced in the evening plasma collections from Wistar Han rats.

It is therefore recommended that all blood samples be collected at the same time of day in toxicology studies to allow an appropriate comparison of plasma spectra. Fasting the animals prior to blood collection would not be a recommended alternative. It is not a common procedure in toxicology studies and could result in alterations in other small molecules as a result of stress. Randomisation of the sample collection procedure is recommended when taking samples from a large study in order to avoid systematic errors.

Acknowledgements

The authors acknowledge GlaxoSmithKline Pharmaceuticals (UK) for the funding of this work. SD also received the financial support of the EU as part of the Marie Curie Host Fellowship Programme.

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