Biochemical Pharmacology, Vol. 46, No. 5, pp. 953–957, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

SHORT COMMUNICATIONS

Induction of 5-oxoprolinuria in the rat following chronic feeding with *N*-acetyl 4-aminophenol (paracetamol)

(*Received* 30 *April* 1993; *accepted* 24 *May* 1993)

Abstract—The urine of rats fed on 1% paracetamol in the diet for up to 10 weeks was analysed using 500 MHz ^{1}H NMR spectroscopy. After 3 weeks, paracetamol-dosed rats were found to excrete massive quantities of an unknown metabolite in the urine. Using a range of 1 and 2 dimensional ^{1}H NMR spectroscopic techniques, solid phase extraction and mass spectrometry, the metabolite was identified at 5-oxoproline (5OXP, pyroglutamic acid). Rats fed paracetamol plus methionine, which prevents the depletion of sulphur-containing amino acids, did not develop 5OXP-uria during the study period. Quantitative ^{1}H NMR spectroscopy of whole urine showed that no 5OXP appeared in the urine in the first 2 weeks of feeding paracetamol to the animals, but, urinary concentrations then rose rapidly up to 1 M in some animals. This unusually high concentration of 5OXP in the urine and its prevention by methionine indicates that chronic high level paracetamol dosing leads to severe depletion of sulphur-containing amino acids including cysteine with consequent disruption of the glutathione cycle.

The biochemical and pathological effects of acute paracetamol (*N*-acetyl 4-aminophenol, acetaminophen) overdose are well understood, but the effects of chronic paracetamol dosing are less well known [1]. The currently accepted mechanism of acute paracetamol toxicity involves formation of a reactive metabolite and its reaction with and consequent depletion of hepatic glutathione (GSH*). Following this, further amounts of reactive metabolites covalently bind to cellular proteins as a consequence of lowered hepatic GSH [1]. In a recent study of the chronic effects of paracetamol it was shown that rats given 1% paracetamol in the diet stopped growing and this inhibition of growth could be reversed or prevented by adding 0.5% methionine to the diet [2]. It seems likely that this effect is due to increased utilization of endogenous sulphur-containing amino acids to form the sulphate and cysteine conjugates of paracetamol which are extensively excreted in the urine. This may occur to such an extent that the animals become effectively depleted of the essential sulphur amino acids with consequent deleterious effects on protein metabolism and growth [2]. In order to investigate the biochemical basis of this phenomenon further we collected urine from animals fed diets containing paracetamol and paracetamol with D/L methionine, and used high field ^{1}H NMR spectroscopy to simultaneously measure the urinary excretion of paracetamol metabolites and endogenous low molecular weight (MW) organic compounds. Previous studies have established that high resolution ^{1}H NMR spectroscopy of biofluids is a powerful tool with which to explore the metabolism and possible toxicological effects of drugs and foreign compounds [3–8]. We have previously reported the use of 500 MHz ^{1}H NMR spectroscopy for the quantitative study of the excretion of paracetamol in normal subjects receiving therapeutic doses and in subjects suffering from paracetamol overdose [9, 10]. We have also demonstrated the use of high field 2 dimensional ^{1}H–^{1}H correlation spectroscopy as a signal assignment aid for the partially overlapped proton resonances of paracetamol metabolites in urine [11]. The aim of this preliminary ^{1}H NMR spectroscopic investigation was to correlate the metabolic fate of the dosed paracetamol in rats with changes in the endogenous metabolite excretion profile in order to gain insight into the biochemical and toxicological effects of chronic paracetamol treatment. Our studies have led to the discovery of massive 5-oxoprolinuria as a consequence of chronic paracetamol feeding in the rat, a phenomenon that may have considerable clinical and toxicological significance.

Materials and Methods

Animals and dosing. Young male Wistar rats (Olac, Bicester, U.K.) weighing about 120 g, were fed on a diet containing 15% Casein (as previously described, [3]) for 1–6 weeks and again at 10 weeks. Two groups of three rats were given the same diet with or without the addition of 1% paracetamol (Sigma Chemical Co., London, U.K.) with or without 0.5% of L-methionine (Sigma). Rats were weighed every other day and urine was collected at the end of each week, by placing three rats together in a metabolism cage for 6 hr. Urine samples were collected over ice in a tube containing a crystal of thymol as a preservative.

Sample preparation. Urine samples were freeze-dried and reconstituted with their original volumes of ^2H_2O prior to NMR measurement, or were untreated except for the addition of ^2H_2O (final concentration 10% ^2H_2O) to provide an internal field frequency lock. An internal chemical shift [δ 0 ppm] and concentration reference was also present (dissolved in the ^2H_2O) in the form of sodium-3-(trimethylsilyl) [^2H]$_4$ propionate (TSP) at a final concentration of 2.28 mM.

^{1}H *NMR spectroscopy of urine.* Single pulse ^{1}H NMR spectra were measured using a JEOL GSX 500 spectrometer operating at 500 MHz ^{1}H resonance frequency. All spectra were recorded at 293 K and were the result of 128 free induction decays (FID's) following 45° pulses collected into 32,768 computer points using a 6000 Hz spectral width. A total pulse recycle time of 6 sec was used to allow T_1 relaxation of the urinary metabolite protons. Exponential line broadening functions of 0.2 Hz were applied to the FID's prior to Fourier transformation (FT) to improve the

* Abbreviations: 5OXP, 5-oxoproline; GSH, reduced glutathione; FID, free induction decay; TSP, sodium tetradeuterio trimethylsilylpropionate; DQCOSY, double quantum filtered phase sensitive correlation spectroscopy; FAB, fast atom bombardment; SPEC-NMR, solid phase extraction chromatography with NMR detection; FT, Fourier transformation.

signal to noise ratio. The residual water signal was suppressed by a secondary irradiation field applied at the water resonance frequency, the power being gated off during acquisition. Quantitative data were obtained by comparing the signal intensity of selected drug metabolite and endogenous metabolite resonances with those of the added TSP standard.

500 MHz 2 dimensional phase-sensitive double quantum filtered homonuclear correlation spectroscopy (DQCOSY, [12]). DQCOSY experiments were performed on selected urine samples to aid identification of novel metabolites. The DQCOSY experiments results in a contour plot of the chemical shifts in two frequency domains with off-diagonal elements correlating the chemical shifts of spin–spin coupled protons (separated by no more than four single bonds). The DQCOSY experiment only allows magnetization decay for double or higher order quantum transitions to be measured, so that singlets are eliminated from the 2 dimensional matrix. The DQCOSY acquisition conditions included a 2.7 sec T_1 relaxation delay between successive pulse cycles; the mixing period was varied in 256 increments giving 256 F_1 data points which were then zero-filled to 1024 data points prior to FT. The FIDs (16 scans per increment) were collected into 1024 data points in the F_2 frequency domain using a 3600 Hz spectral width. The FIDs were weighted using a sinebell function prior to FT. The final FT data matrix [1024 × 1024 points] being symmetrical was mathematically symmetrized prior to plotting using standard spectrometer software.

NMR monitored solid-phase extraction chromatography and mass spectrometry. Samples were also subjected to solid-phase extraction with NMR detection (SPEC-NMR [6, 7]) experiments in order to partially purify and identify selected metabolites. Samples of whole urine were loaded onto a preconditioned Bond-Elut™ column (Analytichem International, Harbor City, CA, U.S.A.) containing a novel mixed multimodal phase with anionic, cationic and non polar separation capabilities (report in preparation). This phase is capable of the simultaneous extraction of acidic, basic and neutral compounds. The solid-phase extraction column was eluted sequentially with HCl/methanol, ammoniacal methanol and 100% methanol. Solvent was removed using a stream of N_2 and freeze-drying before re-dissolving in ^2H_2O prior to NMR analysis. Mass spectra of solid-phase extracts were obtained by fast atom bombardment (FAB) mass spectrometry in both positive and negative-ion modes using a Kratos MS80RF mass spectrometer with a DS55 data system. Glycerol was used as the FAB matrix.

Results

^{1}H NMR urinalysis. The stacked ^{1}H NMR spectra of control rat urine (Fig. 1a) and those from rats treated with 1% paracetamol and 1% paracetamol plus 1% methionine in the diet for a period of 10 weeks are shown in Fig. 1b and c, respectively. We have previously assigned the ^{1}H NMR signals from many of the endogenous metabolites present in normal human and rat urine [6, 7] and all major paracetamol metabolites in human urine [9, 10]. The chemical shifts and spin–spin coupling patterns of all the major paracetamol metabolites have been reported previously [9] and several of the signals (particularly those from the *N*-acetyl methyl groups) from paracetamol β_1-D-glucuronide, paracetamol-*O*-sulphate, and the *N*-acetyl cysteine conjugate of paracetamol were particularly obvious in the 1 dimensional spectra of urine (Fig. 1) from rats dosed with paracetamol and were readily quantified from their respective *N*-acetyl signals after spectral expansion and mild resolution enhancement (Table 1). We have previously discussed the use of ^{1}H NMR spectroscopy in the quantitative determination of paracetamol metabolites in human urine following single therapeutic doses [9]. In addition to paracetamol-related signals in the urine of rats fed on the paracetamol containing diet for 3 weeks or more, there were four large additional multiplet ^{1}H resonances at δs 2.05, 2.41, 2.51 and 4.18 in the ratio 1:2:1:1 (Fig. 1) which were not present in control rat urine. These signals were also absent at earlier time-points for the paracetamol-treated rats and were not present in the urine of control rats or those given paracetamol and methionine at any time during the experimental period.

Identification of 5-oxoproline (5-OXP) in urine of paracetamol-dosed rats. Two-dimensional ^{1}H–^{1}H DQCOSY spectroscopy of urine from the paracetamol-treated rats clearly showed that the signals from the unknown compound had a characteristic and strong ^{1}H–^{1}H connectivity pattern proving that the signals were from protons on the same molecule and establishing their "nearest neighbour" pattern. The chemical shifts, signal intensities and connectivities indicated that the molecule contained a methylene (CH_2 triplet, δ 2.41) group probably with an adjacent carbonyl function from the chemical shift. This methylene group is coupled to two other strongly-coupled highly non-equivalent methylene protons (second order spin system, δ 2.05 and 2.51). These protons were in turn both coupled and hence adjacent to a methine (CH) group (X of ABX spin system, δ 4.18) similar in chemical shift to an α-CH proton of an amino acid. To obtain more structural information on the unknown, urine from a paracetamol-treated rat was fractionated using a SPEC-NMR procedure. Using the mixed phase (anionic, cationic and C_{18}) solid phase extraction procedure outlined above, the unknown material was partially purified and obtained in the 100% methanol fraction (confirmed by rerunning the ^{1}H NMR spectrum of the extract). The methanolic extract containing the unknown was then subjected to negative-ion FAB mass spectrometry which gave an indicated molecular mass of 129 Da. This information taken together with the ^{1}H NMR data indicated that the unknown was 5OXP. Subsequent spiking of the samples with authentic 5OXP and remeasurement of the ^{1}H NMR spectra confirmed the signal assignment of 5OXP (Fig. 1). The concentrations of 5OXP were then measured in the urine samples of the paracetamol treated rats by integration of the ^{1}H signal at δ 2.41 in the single pulse ^{1}H NMR spectra and comparison with the signal intensity of the TSP standard. The detection limit for 5OXP in these samples under the given instrument conditions was about 20 nM/mL [confirmed by spiking experiments]. The urinary concentrations and ratios of paracetamol metabolites and 5OXP concentrations and ratios in paracetamol and paracetamol plus methionine treated rats are given in Table 1. The amount of 5OXP excreted greatly exceeded the paracetamol intake and was present in concentrations up to 1 M in animals fed on 1% paracetamol for more than 2 weeks. In control rats and rats fed on 1% paracetamol plus 1% methionine, 5OXP was not detectable in the urine by NMR at any urine collection time-point.

The conventional chemical derivatization/gas chromatographic methods for detecting 5OXP in urine are laborious and time-consuming and would normally only be used if its presence was considered likely. Here, the use of ^{1}H NMR enabled the facile detection of 5OXP even though no deliberate search for this compound was being undertaken. As such, the results presented here provide a graphic illustration of the power of high resolution ^{1}H NMR spectroscopy as an exploratory analytical tool and as a means of investigating poorly understood toxicological phenomena.

Discussion

5-Oxoprolinuria has been detected as a very rare inborn error of metabolism in humans (there are perhaps no more than 50 known cases in the world) where there is either a deficiency of 5-oxoprolinase or GSH-synthetase, leading to elevated levels of 5OXP in the blood and urine [13]; it

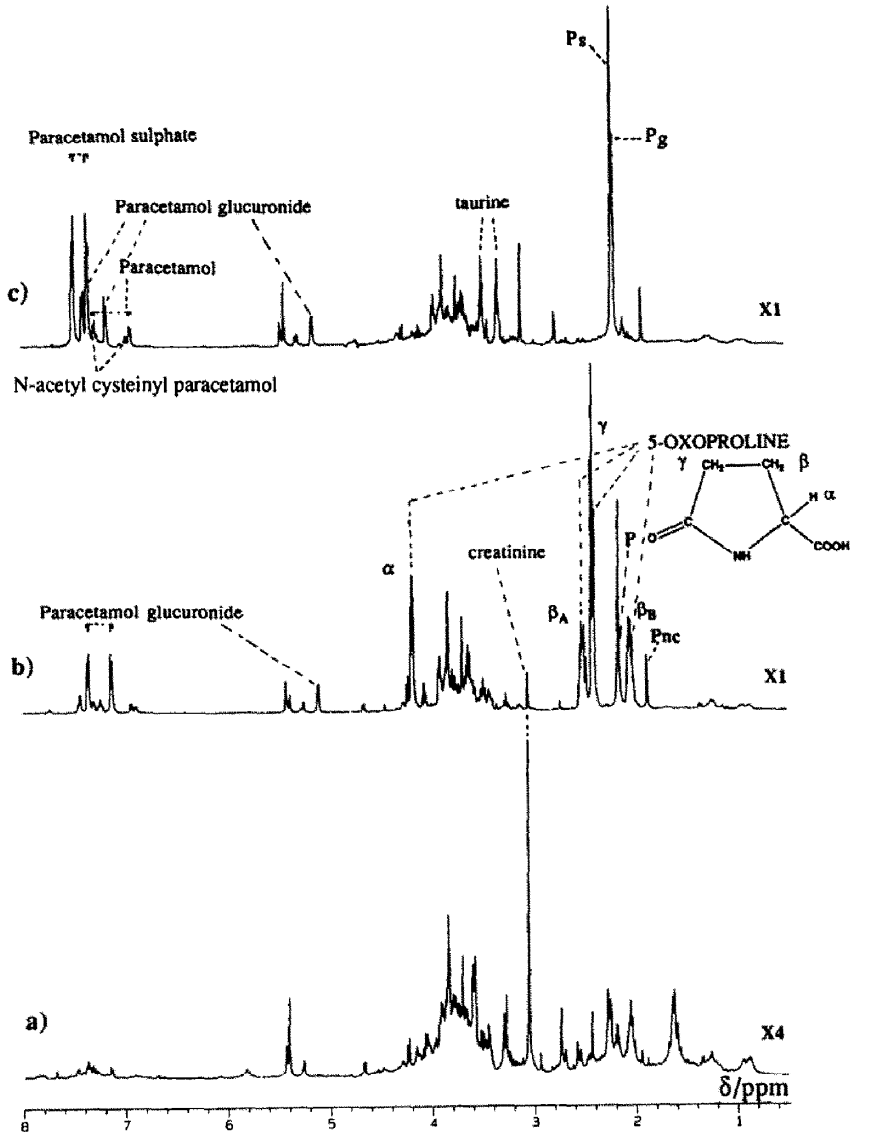

Fig. 1. Single pulse 500 MHz ^{1}H NMR spectra of 6 hr pooled urine collection from (a) control rats (×4 vertical expansion) (b) rats fed on 1% paracetamol diet for 10 weeks (×1 vertical expansion) and (c) rats fed on 1% paracetamol + 1% methionine diet for 10 weeks (×1 vertical expansion). Key: P, Pg and Ps, *N*-acetyl signals of paracetamol, paracetamol glucuronide and paracetamol sulphate, respectively (the resolved aromatic signals of these and the parent drug are labelled separately), Pnc is the resolved cysteinyl side chain *N*-acetyl group.

has not been reported in rats. 5OXP is produced during the cyclic metabolism of GSH to cysteine glutamate and glycine (Fig. 2). Normally 5OXP is hydrolysed by 5-oxoprolinase to glutamate which is then reutilized [13]. 5-Oxoprolinuria is usually caused by a deficiency of glutathione synthetase (EC 6.3.2.3) and much more rarely a deficiency of 5-oxoprolinase (EC 3.5.2.9). One previous case report identified a patient with massive 5-oxoprolinuria but in which neither of the enzymes were thought to be deficient and a possible exogenous dietary toxic agent was suspected but unidentified [14]. Another recent communication indicated that mild, but statistically significant, 5-oxoprolinuria occurs in humans following paracetamol treatment [15]. We have reported here that massive 5-oxoprolinuria can be induced in experimental rats fed chronically on casein diets containing 1% paracetamol. It is important to note that co-administration of methionine with the paracetamol not only completely protects against the 5-oxoprolinuria but also against the severe weight loss found in paracetamol treated rats [2]. As methionine can act as the sulphur source both for inorganic sulphate and cysteine production and GSH generation we hypothesize that the 5-oxoprolinuria is essentially a result of a lack of sufficient dietary sulphur containing amino acids to compensate for the loss of sulphur as paracetamol metabolites. In the present study the quantities of 5OXP excreted by paracetamol-only treated rats were far greater than the quantities of excreted paracetamol metabolites. This indicates that 5OXP cannot arise solely as a result of stochiometrically-increased

Table 1. Concentrations in mM and ratios of 5OXP, paracetamol and its *O*-glucuronide, *O*-sulphate and *N*-acetyl-cysteine conjugates, measured by 500 MHz ^{1}H NMR spectroscopy in the weekly collected 6 hr pooled urine of rats (N = 3) fed on 1% paracetamol

Study week	Paracetamol	Glucuronide	Sulphate	*N*-Acetyl-cysteine	5OXP
1	0.37	3.42	7.03	1.20	0
	1	**9**	**19**	**3**	**0**
2	3.62	27.56	22.72	6.04	0
	1	**8**	**6**	**2**	**0**
3	2.99	22.8	12.51	3.80	105.0
	1	**8**	**4**	**1**	**35**
4	1.03	3.97	3.06	0	51.3
	1	**4**	**3**	**0**	**50**
5	1.61	12.72	6.83	2.34	57.0
	1	**8**	**4**	**1**	**35**
6	1.84	10.69	7.99	0	103.5
	1	**6**	**4**	**0**	**56**
10	17.95	169.97	40.7	27.5	1094
	1	**9**	**2**	**2**	**61**

Figures in bold represent molar ratios to urinary paracetamol (unchanged drug).

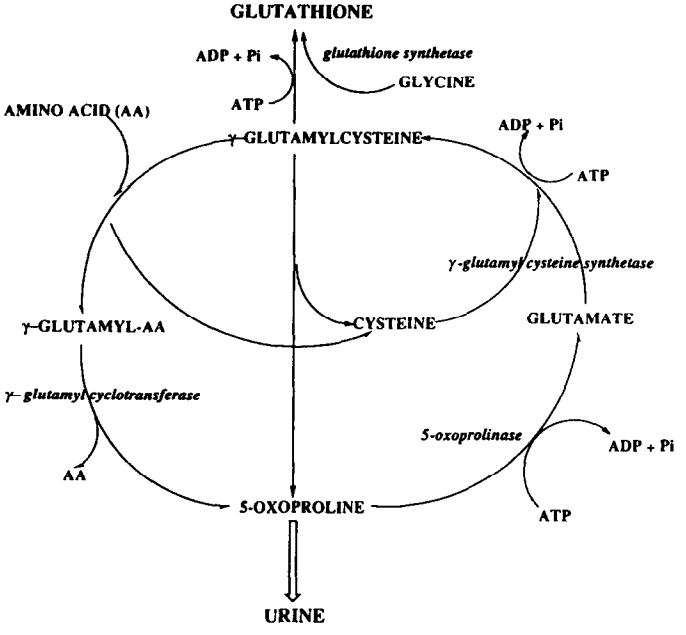

Fig. 2. The γ-glutamyl cycle in 5-oxoprolinuria.

generation of 5OXP during the metabolism of paracetamol to its cysteine conjugate after the GSH adduct has formed from the oxidized metabolites of paracetamol. If we assume that a rat consumes about 10% of its weight daily this would give an intake of 1 g (6.6 mmol) of paracetamol per day per kg of rat. At the same time the excretion of 5OXP was about 5 mM/day/kg. However, at most only 25–30% of the paracetamol was metabolized via GSH to the cysteine conjugates and in consequence the bulk of the 5OXP must arise via another pathway. The observed 5-oxoprolinuria cannot be due to the direct inhibition of 5-oxoprolinase by paracetamol, since any such inhibitory effect would be found in the first few days of feeding the diet and not take 2–3 weeks to appear. The most likely route in metabolism through which excessive levels of 5OXP could arise is in a hitherto unknown inter-relation between GSH breakdown products and the availability of cysteine for re-synthesis of GSH. Since 5OXP is generated during the normal turnover of GSH during amino acid transport, there is scope for accumulation of 5OXP if this compound is generally removed not by the action of 5-oxoprolinase (leading to glutamate), but directly into the GSH cycle by conjugation with cysteine. In the absence of sufficient cysteine (due to a generalized depletion of sulphur containing compounds sequestered in paracetamol conjugation) the quantities of 5OXP produced are in excess of the capacity of 5-oxoprolinase for catabolism and hence 5OXP accumulates and overflows into the urine. The high concentrations of

5OXP in these animals are also consistent with the loss of a significant proportion of the animals' total daily amino acid input in the diet with the consequent weight loss effects. We are currently investigating these phenomena further, but the immediate implication of this work is that there may be a possibility of sulphur amino acid depletion in patients on low protein diets taking paracetamol for long periods and that 5-oxoprolinuria may be a consequence and possible indicator of the deficiency.

Acknowledgement—We thank Sterling Winthrop for financial support.

FARIDA Y. K. GHAURI*
ANDRE E. M. MCLEAN†
DENIS BEALES†
IAN D. WILSON‡
JEREMY K. NICHOLSON*§

*Department of Chemistry
Birkbeck College
University of London
Gordon House
29, Gordon Square
London WC1H 0PP
†Toxicology Laboratory
Department of Clinical Pharmacology
School of Medicine
University College
University of London
5, University Street
London WC1E 6JJ
‡Department of Safety of Medicines
Zeneca Pharmaceuticals
Mereside
Alderley Park
Macclesfield
Cheshire SK10 4TG
U.K.

REFERENCES

1. Nelson S, Molecular mechanisms of the hepatotoxicity caused by acetaminophen. *Semin Liver Dis* **10**: 267–278, 1990.
2. Maclean AEM, Armstrong GR and Beales D, Effect of D or L methionine and cysteine on the growth inhibitory effects of feeding 1% paracetamol to rats. *Biochem Pharmacol* **38**: 347–352, 1989.
3. Nicholson JK and Wilson ID, High Resolution Proton NMR spectroscopy of biological fluids. *Prog NMR Spectrosc* **21**: 449–501, 1989.
4. Bales JR, Higham DP, Howe I, Nicholson JK and Sadler PJ, Use of high resolution proton nuclear magnetic resonance spectroscopy for rapid multi-component analysis of urine. *Clin Chem* **30**: 426–432, 1984.
5. Gartland KPR, Bonner F and Nicholson JK, Investigations into the biochemical effects of region-specific nephrotoxins. *Mol Pharmacol* **35**: 242–251, 1989.
6. Wilson ID and Nicholson JK, Solid Phase Extraction Chromatography and NMR spectroscopy [SPEC-NMR] for the analysis of drug metabolites in urine. *J Pharm Biomed Anal* **6**: 151–165, 1988.
7. Wilson ID and Nicholson JK, Solid Phase Extraction Chromatography and NMR spectrometry for the detection and identification of drug metabolites in urine. *Anal Chem* **59**: 2830–2832, 1987.
8. Nicholson JK, Timbrell JA and Sadler PJ, Proton NMR spectra of urine as indicators of renal damage: mercury nephrotoxicity in rats. *Mol Pharmacol* **27**: 644–651, 1985.
9. Bales JR, Sadler PJ, Nicholson JK and Timbrell JA, Study of the urinary excretion of acetaminophen and its metabolites by proton NMR spectroscopy. *Clin Chem* **30**: 1631–1636, 1984.
10. Bales JR, Bell JD, Nicholson JK, Sadler PJ, Timbrell JA, Hughes RD, Bennett PN and Williams R, Metabolic profiling of body fluids by proton NMR spectroscopy: self-poisoning episodes with acetaminophen. *Magn Reson Med* **6**: 301–309, 1988.
11. Bales JR, Nicholson JK and Sadler PJ, Two dimensional proton NMR "maps" of acetaminophen metabolites in human urine. *Clin Chem* **31**: 757–762, 1985.
12. Nagayama K, Kumar A, Wuthrich K and Ernst RR, Experimental techniques of two dimensional correlated spectroscopy. *J Magn Reson* **40**: 321–334, 1980.
13. Meister A, 5-Oxoprolinuria [pyroglutamic aciduria] and other disorders of the γ-glutamyl cycle. In: *The Inherited Basis of Metabolic Disease* (Eds. Stanbury JB *et al.*), pp. 348–359. McGraw-Hill Book Company, New York, 1983.
14. Creer MH, Lau BWC, Jones JD and Chan KM, Pyroglutamic acidaemia in an adult patient. *Clin Chem* **35**: 684–686, 1989.
15. Pitt J, Association between paracetamol and pyroglutamic aciduria. *Clin Chem* **36**: 173–174, 1990.

§ Corresponding author.