AGE (2012) 34:181–193
DOI 10.1007/s11357-011-9218-4

Therapeutic paracetamol treatment in older persons induces dietary and metabolic modifications related to sulfur amino acids

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Received: 12 October 2010 / Accepted: 30 January 2011 / Published online: 22 February 2011
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Abstract Sulfur amino acids are determinant for the detoxification of paracetamol (*N*-acetyl-*p*-aminophenol) through sulfate and glutathione conjugations. Long-term paracetamol treatment is common in the elderly, despite a potential cysteine/glutathione deficiency. Detoxification could occur at the expense of anti-oxidative defenses and whole body protein stores in elderly. We tested how older persons satisfy the extra demand in sulfur amino acids induced by long-term paracetamol treatment, focusing on metabolic and nutritional aspects. Effects of 3 g/day paracetamol for 14 days on fasting blood glutathione, plasma amino acids and sulfate, urinary paracetamol metabolites, and urinary metabolomic were studied in independently living older persons (five women, five men, mean (±SEM) age 74±1 years). Dietary intakes were recorded before and at the end of the treatment and ingested sulfur amino acids were evaluated. Fasting blood glutathione, plasma amino acids, and sulfate were unchanged. Urinary nitrogen excretion supported a preservation of whole body proteins, but large-scale urinary metabolomic analysis revealed an oxidation of some sulfur-containing compounds. Dietary protein intake was 13% higher at the end than before paracetamol treatment. Final sulfur amino acid intake reached 37 mg/kg/day. The increase in sulfur amino acid intake corresponded to half of the sulfur excreted in urinary paracetamol conjugates. In conclusion, older persons accommodated to long-term paracetamol treatment by increasing dietary protein intake without any mobilization of body proteins, but with decreased anti-oxidative defenses. The extra demand in sulfur amino acids led to a consumption far above the corresponding population-safe recommendation.

This paper is dedicated to Christiane Obled (retired), who had the idea of the study.

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Keywords Detoxification · Glutathione · Metabolomic · Older persons · Paracetamol · Sulfur amino acids requirement

Introduction

Paracetamol (*N*-acetyl-*p*-aminophenol) is probably the most widely used of all analgesics in the world (Prescott 2005). It is commonly prescribed for long-term therapy in elderly, for joint problems such as arthritis and gout (Chen et al. 1985). It is considered as a safe drug, which is mainly eliminated in urines (85% to 95%; Forrest et al. 1982; Critchley et al. 1986). A small part is excreted unchanged (5%); the major part is excreted as its glucuronide conjugate (55%) followed by its sulfate conjugate (30%). Glucuronide and sulfate conjugations are referred as phase II reactions. The remaining is converted to *N*-acetyl-*p*-benzoquinone imine (NAPQI) by the cytochrome P-450 (CYP450) oxidase enzyme system, a phase I reaction. This toxic metabolite is rendered harmless by reduced glutathione (GSH, γ-glutamyl-cysteinyl-glycine), the main intracellular anti-oxidant compound. Degradation of GSH-paracetamol conjugate leads to cysteine paracetamol (free, sulfate and glucuronide forms) and *N*-acetylcysteine paracetamol (mercapturic acid of NAPQI; Hart et al. 1982). The metabolism of one single dose of 1 g paracetamol appears similar in young adults (21 years) and in elderly (79 years) man; nevertheless, the partial metabolic clearance of sulfate conjugate and the renal clearance of unchanged paracetamol is reduced in elderly (Miners et al. 1988). This could be related not only to aging-associated low capacity to detoxify drugs (Klotz 2009) but also to the cysteine/GSH deficiency syndrome observed in elderly (Maher 2005; Dröge 2005; Mercier et al. 2006).

Beyond the need of cysteine for protein synthesis, it is required for other syntheses such as glutathione and sulfate. Cysteine is provided by dietary protein intake, its release from body proteins and from glutathione, and also by its endogenous synthesis from methionine and serine through the transsulfuration pathway. When the endogenous disposal of cysteine is insufficient regarding its metabolic utilizations, it becomes an indispensable amino acid (Obled et al. 2004). Considering that about 40% of paracetamol is metabolized as sulfur-containing compounds, the maximal therapeutic dose of paracetamol (4 g/day) induces a theoretical extra demand of 1.3 g/day cysteine (i.e., 18 mg/kg/day). This is equivalent to an extra demand of 23 mg/kg/day methionine (stoichiometry). This amount is obviously high regarding the recommended population-safe intake of methionine, which has been evaluated at 21–27 mg/kg/day in the absence of cysteine intake (Di Buono et al. 2001; Kurpad et al. 2003). The prevalence of protein-energy malnutrition increases with age and reaches 4–10% in older persons living at home, 15–38% in institutionalized persons and 30–70% in hospitalized ones (Raynaud et al. 2007). Cysteine requirement might be uncovered in long- term paracetamol-treated older persons, potentially leading to metabolic deteriorations.

Previous studies in animal models and adult persons show that short and long-term paracetamol treatments affects sulfur amino acid and glutathione metabolisms. One single dose of paracetamol (2 g) decreases plasma cysteine and GSH concentrations (Burgunder et al. 1989) and 1 g/day paracetamol oxidizes the plasma cysteine/cystine redox potential (Mannery et al. 2010) in young adult men. Paracetamol induces dose-dependent stimulations of rates of turnover of cysteine and glutathione in men (Lauterburg and Mitchell 1987). Increasing the dose from 0.5 to 3 g in adults leads to a decrease in the partial metabolic clearances of sulfate and glutathione conjugates, likely due to the depletion of hepatic pools of GSH and sulfur donor (3′-phosphoadenosine 5′-phosphosulfate, whose precursor is inorganic sulfate) (Slattery et al. 1987). After long-term paracetamol treatment, GSH concentration decreased in rat liver (Buttar et al. 1977) and in human plasma (Trenti et al. 1992). Such a decrease should be avoided in elderly, since a decrease in plasma or blood red cells GSH concentration is associated with a lower physical and cognitive health in that population (Julius et al. 1994). Long-term paracetamol treatment increases sulfur amino acid requirement. Indeed, rats and mice chronically fed paracetamol failed to grow, this inhibition being dose-dependent and restored by the addition of methionine or cysteine to the diet (allowing protein accretion) (Reicks and Hathcock 1989; McLean et al. 1989). Aging aggravates paracetamol-induced alterations: in comparison to young or adult mice, liver GSH was low in old mice, and old mice fail to normalize liver and kidney GSH concentrations after a single administration of paracetamol (Chen et al. 1990; Richie et al. 1992; Chen et al. 2000).

Older persons under long-term paracetamol treatment appear to be at risk for nutritional and metabolic

troubles. To our knowledge, that point has not been investigated, despite the known bad impact of high drug intake on nutritional status, especially in the frail ones (Chen et al. 1985; Pickering 2004). We hypothesized that usual sulfur amino acid intake of older persons could be insufficient to support both physiological needs and paracetamol detoxification. If they failed to adapt through an increase in protein intake, deleterious alterations in paracetamol metabolism, glutathione status, and/or whole body protein stores would occur and contribute to the development of the frailty syndrome. The aim of the study was to study how older persons adjust to a long-term paracetamol treatment. The main targets were dietary intake, paracetamol metabolism, sulfur amino acid-related metabolism, urinary excretion of sulfur and nitrogen, and also overall metabolism assessed by urinary metabolomic.

Subjects and methods

Subjects

Eleven persons aged >69 years suffering from arthritic pain, and who were planned to be administered paracetamol as an analgesic, were selected to participate in the study by their physicians. The study was then performed by the Clinical Trials Investigation Center. The main exclusion criterion was an ongoing treatment with *N*-acetylcysteine. The study protocol was approved by the local ethical committee, and all volunteers signed an informed consent form before the inclusion in the study. One subject was excluded a posteriori because urine analysis revealed paracetamol ingestion at baseline. This study has been registered as ClinicalTrial.gov NCT01116596.

Study design

The subjects were treated with 3 g/day paracetamol for 14 days. They had not received any dietary recommendations. At baseline and at d14, fasting blood samples were collected into heparin-containing tube (5 ml) for paracetamol quantification and into EDTA-containing tube (5 ml) for other measurements. The former was centrifuged, and plasma was frozen at −20°C until measurement. An aliquot of the latter was rapidly collected and frozen at −80°C until blood glutathione quantification. Plasma was separated from the remaining EDTA-blood then frozen at −80°C until sulfate and amino acid quantifications. Urine was collected at baseline (second morning urine) and 24-h urines were collected on the fourteenth day of treatment for urinary paracetamol metabolites quantification, large-scale metabolomic analysis, total nitrogen and total sulfur quantifications. Two 10-ml aliquots of each urine sample were frozen at −20°C until analysis. Dietary intakes were collected before and at the end of the treatment.

Dietary recordings

The 2 days before the beginning of the treatment and the four last days of treatment, the volunteers had to record their food intakes in a notebook. These data have been checked with a dietician with the validated photo album SUVIMAX (Hercberg et al. 1994; Le Moullec et al. 1996). Then, the conversion of food data in nutrients intakes has been performed using the software Geni (Geni version 6.0, Micro6—Villers les Nancy, France), which includes the dietary composition table REGAL (Favier et al. 1995). The dietary intake in methionine plus cysteine has been evaluated considering that proteins consumed by the French population contain 35 mg sulfur amino acid/g protein (Patureau Mirand 2003). Sulfur intake derived from methionine and cysteine has been calculated assuming that the methionine intake equals twice the cysteine intake based on dietary protein compositions in amino acids (Block and Weiss 1956).

Blood and plasma analyses

Plasma paracetamol concentrations were measured by use of HPLC with a diode array detection and a limit of quantification of 0.05 μg/ml (Di Pietra et al. 1996). Total blood glutathione (reduced and oxidized) concentration was quantified spectrophotometrically using a standard enzymatic recycling procedure and 5,5'-dithio-bis-2-nitrobenzoic acid as oxidant (Malmezat et al. 1998). Plasma sulfate concentration was determined by turbidimetry using the QuantiChromTM sulfate assay kit (Gentaur, Paris, France). Total non-protein cysteine (cysteine plus cystine plus cysteine bound to proteins via disulfide bridges) was measured by colorimetry in plasma treated with dithiothreitol before deproteinization (Gaitonde 1967; Malloy et al. 1981). Plasma-free amino acids were quantified by ion-exchange chromatography with post-column ninhydrin

detection (Bio-Tek Instrument, Serlabo Technologies, Entraigues sur la Sorgue, France).

Quantification of urinary paracetamol metabolites

Paracetamol and its sulfate conjugate were purchased from Sigma Aldrich (L'Isle d'Abeau, France), glucuronide paracetamol from Chemos GmBH (Regenstauf, Germany), *N*-acetylcysteine paracetamol and cysteine paracetamol from Toronto Research Chemical (Brisbane Road, Toronto, Canada). All these compounds were quantified in urine using a liquid chromatography (LC) Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) coupled to a triple-quadrupole mass spectrometer (MS) API 2000 (Applied Biosystems/MDS Sciex, Foster City, CA, USA), equipped with an electrospray ionization source interface operating in the positive mode. MS parameters were optimized by direct infusion of standard solutions. Maximum sensitivity was obtained for a capillary voltage at 5,000 V, a source temperature at 450°C, and GS1 and GS2 at 40 and 60, respectively. Each standard was optimized one by one to obtain the best sensitivity.

Urine samples were tenfold diluted in water with 1 μg/ml of 4-4 amido phenyl aminothiazole as internal standard. Injection volume was set at 10 μl. Chromatographic separation was performed on an 150×2.1 mm Atlantis dC18 column (Waters, Saint Quentin en Yvelines, France), with particle size of 5 μm at a flow rate of 200 μl/min. Urine samples were eluted from the LC column using the following gradient: 0 to 5 min: 90% A; 5 to 15 min: 10 to 100% B, 15 to 25 min at 90% A. Solvent A was water containing 0.01 mM ammonium formiate, and solvent B was methanol.

Quantification was achieved by measuring product ions (multiple reaction monitoring [MRM]) from the fragmentation of the protonated [M+H]+molecules. The collision energy potential and the collision exit potential were then adjusted to optimize the signal for the most abundant product (daughter) ions (*m*/*z* 152>110 for paracetamol, *m*/*z* 271>140 for cysteine paracetamol, *m*/*z* 313>140 for *N*-acetylcysteine paracetamol, *m*/*z* 152>110 for glucuronide paracetamol and sulfate paracetamol and *m*/*z* 234>192 for the internal standard) using nitrogen as the collision gas at a pressure of 10^{-3} mbar. Quantification was performed using calibration curves with external standards. The mean variability of each standard across its calibration curve (%) was 8.2±4.7 for paracetamol, 7.9±5.8 for cysteine paracetamol, 14.2±7.5 for *N*-acetylcysteine paracetamol, 13.9±10.2 for glucuronide paracetamol, and 6.7±4.3 for sulfate paracetamol.

Glucuronide and sulfate conjugates of cysteine paracetamol were not commercially available. Therefore, total free form was quantified after an enzymatic hydrolysis of 40 μl of 10-time diluted urine with 20 μl of *Escherichia coli* β-glucuronidase sulfatase (Sigma Aldrich—L'Isle d'Abeau, France) in buffered medium (0.2 M sodium acetate, pH 5) for 1.5 h, at 37°C. The reaction mixture was then stopped with 140 μl of methanol and centrifuged at 3,900×*g* for 5 min. Twenty microliters of 10 μg/ml 4-4 amido phenyl aminothiazole was added to 50 μl of the supernatant and diluted in 200 μl water. Quantifications of paracetamol, cysteine paracetamol, and *N*-acetylcysteine paracetamol were performed in hydrolyzed urines as described above. The enzymatic procedure was validated by the fact that paracetamol in hydrolyzed urine (1.06±0.14 mg/ml) was not significantly different from the sum of free paracetamol and its sulfate and glucuronide conjugates (1.11±0.16 mg/ml).

Metabolomics

Urinary metabolic profiles were determined by LC-QToF using a metabolomic approach. This untargeted method consisting in the simultaneous analysis of a high number of metabolites allows determining most of metabolic perturbations.

The urine samples (500 μl) were defrosted at room temperature, centrifuged at 7,000×*g* for 5 min at 4°C, and then diluted fourfold with distilled water. Chromatography was performed with a Waters Acquity UPLC system (Waters Corporation, Manchester, United Kingdom). The UPLC system was coupled to a Waters Qtof-Micro equipped with an electrospray source and a lockmass sprayer. The source temperature was set to 120°C with a cone gas flow of 50 l/h, a desolvation temperature of 300°C, and a nebulization gas flow of 400 l/h. The capillary voltage was set at 3,000 V and the cone voltage to 30 V. The mass spectrometric data were collected in continuum full-scan mode with a mass-to-charge ratio (*m*/*z*) of 100–1,000 from 0 to 22 min, in positive and negative modes. All analyses were acquired by using the lockspray with a frequency of 5 s to ensure accuracy. Leucine enkephalin was used as the lock mass at a concentration of 0.5 ng/l (in MeOH/water, 50/50 by vol with 0.1% formic acid).

To avoid possible differences between sample batches, a Latin square was carried out to obtain a randomized list of samples for analysis. Separation was carried out at 30°C using a 2.1×100 mm Acquity UPLC BEH Shield RP18 column (Waters), with a particle size of 1.7 μm at a flow rate of 0.4 ml/min. Sample was eluted from the column using the following gradient: 0 to 2 min: 100% A; 2 to 7 min: 0 to 10% B; 7 to 22 min: 95% B. Solvent A was water and solvent B was acetonitrile, both solvents containing 0.1% formic acid. Injection volume was set to 8 μl.

The raw data were transformed to centroid mode and mass corrected before being analyzed with MarkerLynx Applications Manager v1.0. The liquid chromatography–MS data were peak-detected and noise-reduced for both the liquid chromatography and MS components. Each peak in the resulting three-dimensional data set was represented by retention time (RT), *m/z* and its ion intensity in each sample. The matrix obtained was filtered using a Student's *t* test to eliminate the background ions present in blank samples, and then exported for statistical analysis.

Metabolite identification

Metabolites contributing to the discrimination between baseline and d14 samples were identified on the basis of their exact masses which were compared to those registered in Kyoto Encyclopedia of Genes and Genomes database (http://www.genome.jp/kegg/) or in the Human Metabolome Database (www.hmdb.ca) (Wishart et al. 2007) and to those of expected metabolites of paracetamol as described in the literature (Johnson and Plumb 2005; Chen et al. 2008). Identification was confirmed using appropriate standards when available, isotopic patterns and mass fragmentation analyses. For unknowns, the number of plausible elemental compositions may be restricted to a small number (or uniquely identified) with the aid of additional chemical information—e.g., the molecular formula of the parent and knowledge of possible metabolic pathways.

Urinary nitrogen and sulfur

Nitrogen was quantified in thawed urines by the Kjeldahl method (block digestion unit Kjeldatherm and distillation system Vapodest, Gerhardt, Les Essarts le Roi, France) and sulfur was quantified in lyophilized urines by direct combustion and infrared detection (Sulfur SC-144DR analyzer, LECO, Garges-les-Gonesse, France).

Statistical analyses

Data are expressed as means±SEMs. The effect of gender was analyzed using Student's *t* test and the effect of paracetamol treatment using Student's *t* test for paired data. Statistical significance was set at $P<0.05$ and analyses were performed with StatView for Windows, version 5 software (SAS Institute, Cary, NC).

Each ion obtained in the metabolomic data matrix was tested for its significance. An ion was considered significant if the Student's *t* test for paired data comparing baseline and d14 samples ($P\leq0.05$).

Results

Subjects

Six women and five men were included in the study but one woman was excluded a posteriori because paracetamol metabolites were detected in the urine collected before the treatment. The anthropometric characteristics (Table 1) were not different between women and men (expect a trend for a higher height in men). None of other studied parameters was dependent on gender; therefore, all subjects were pooled. Subjects mean (±SEM, $n=10$) age was 74.0±1.2 years, body weight 74.2±3.6 kg, height 165±3 cm and body mass index 27.2±1.1 kg/m^2. All of them were suffering from joint pain treated with paracetamol. Half of the patients had other drugs. These treatments

Table 1 Baseline characteristics of subjects

	Female ($n=5$)	Male ($n=5$)	All ($n=10$)
Age (year)	75.8±0.9	72.2±2.0	74.0±1.2
Body weight (kg)	73.2±7.0	75.1±2.9	74.2±3.6
Height (cm)	160±4	170±3 *	165±3
Body mass index (kg/m^2)	28.4±2.2	26.0±0.5	27.2±1.1

Values are means±SEM

*$P<0.1$ (Student's *t* test). Trend to be different from female values

had been installed at least for the last 2 months and no new drug was introduced during the course of the protocol. The drug metabolized by one of the CYP450 variants of paracetamol metabolism were lansoprazole (CYP3A), pantoprazol (CYP3A4), amlodipine (CYP3A4), acénocoumarol (CYP1A2), venlafaxine (CYP2D6 and CYP3A4), which is a weak enzymatic inhibitor (no other enzymatic inducer nor inhibitory was noted) (Zhao and Pickering 2011). Only one patient had pravastatine, not significantly metabolized by CYP450. Considering the long-term use of these treatments, they are negligible regarding potential interferences with paracetamol metabolism.

Dietary intakes

Subject total energy intake was unchanged by paracetamol treatment (Table 2); it equaled 23 kcal/kg/day, corresponding to 1.4-time the basal energy metabolism calculated from Harris and Benedict equations (1,233±87 kcal/day). This is consistent with the low level of physical activity of the subjects. The mean amount of alcohol ingested before and at the end of the treatment was low and none subject could be considered at risk of significant interference between alcohol and paracetamol detoxifications (Prescott 2000). Total protein intake was 13% higher at the end (1.06±0.7 g/kg/day) than before (0.93±0.08 g/kg/day) paracetamol treatment. This was mostly explained by an increased intake in animal proteins, which are known to exhibit higher content in sulfur amino acids than vegetable proteins. Indeed, animal protein intake reached at least 42.6±3.3 g/day before treatment and 52.8±3.2 g/day at the end of the treatment, whereas vegetable protein intake attained at least 18.8±1.6 and 20.6±1.7, respectively. Animal and vegetable protein intakes could not be accurately evaluated due to missing values for animal and vegetal protein contents of some foods. Anyway, based on the accurate determination of total protein intakes, the estimated methionine plus cysteine intake and the corresponding sulfur intake were 13% higher at the end than before the treatment.

Blood paracetamol and glutathione, and plasma amino acids and sulfates

Plasma paracetamol was undetectable (lower than 0.05 μg/ml) in baseline and d14 fasting blood samples. Blood concentration of total (reduced and oxidized) GSH was unaffected by paracetamol treatment (Table 3). Plasma total free cysteine (cysteine plus cystine plus cysteine linked to plasma proteins by a disulfide bridge) and sulfates were unchanged by the treatment. Amino acid profile was also unaffected by paracetamol treatment (Table 3).

Urinary paracetamol metabolites, nitrogen, and sulfur at d14

Urinary concentrations and quantities of all paracetamol metabolites excreted at d14 are shown in Table 4. The total amount of paracetamol recovered in 24-h urines was 2.3±0.3 g. The fractional recovery of unchanged paracetamol was 6.4±3.3% and those of paracetamol as conjugates were the highest for glucuronide (65.3±5.6%) followed by sulfate (17.7±3.0%), *N*-acetylcysteine (6.1±0.8%) and cysteine (4.5±0.4%). Cysteine, excreted in all paracetamol conjugates, amounted 177±17 mg/day. Cysteine paracetamol was mainly present as glucuronide and sulfate conjugates but *N*-acetylcysteine paracetamol seemed to be poorly or not conjugated. Sulfur contained in paracetamol sulfate equaled 77±10 mg/day. Sulfur excretion due to overall paracetamol detoxification amounted 124±13 mg/day, whereas total urinary sulfur attained 624±64 mg/day. Total nitrogen excretion amounted 12.4±1.2 g/day and nitrogen urinary concentration was proportional to the sulfur one (nitrogen (g/l)=19.6×sulfur (g/l), r^2=0.87).

Table 2 Dietary intakes

	Baseline	d14
Energy (kcal/day)	1,666±138	1,667±141
Alcohol (g/day)	7.2±4.4	4.8±2.1
Proteins (g/day)	68.4±5.3	77.2±4.8 *
Methionine and cysteine (g/day)	2.40±0.19	2.70±0.17 *
Sulfur from methionine and cysteine (mg/day)	562±44	634±39 *

Values are means±SEM

*P=0.006 (Student's t test for paired values). Significantly different from baseline values

Table 3 Blood glutathione, and plasma sulfate and amino acid concentrations

	Baseline (μmol/l)	d14 (μmol/l)
Glutathione	1,021±79	995±72
Sulfate	382±45	333±32
Cysteine (cysteine+cystine+cysS-Sx)	356±23	353±17
Cystine	52±3	57±3
Serine	70±4	76±3
Taurine	59±4	52±3
Aspartic acid+Asparagine	64±4	71±4
Glutamic acid+Glutamine	655±40	747±30
Alanine	279±31	323±37
Glycine	181±29	193±18
Threonine	81±4	90±5
Phenylalanine	41±2	43±3
Tyrosine	48±4	49±4
Leucine	95±5	105±5
Isoleucine	47±3	53±3
Valine	174±10	188±9
Tryptophane	36±3	37±3
Proline	168±18	175±19
Lysine	127±8	136±6
Histidine	45±3	49±3
Arginine	50±4	53±4
Citrulline	29±3	32±2
Ornithine	46±3	47±3

Values are means±SEM. Methonine was not reported due to its loss during the extraction procedure prior to amino acid analysis. Homocysteine and cystathionine were not quantifiable in present amino acid analyses

Urinary metabolomic

Eleven metabolites acquired in positive and negative modes were detected only at d14 (Table 5), 9 of them were surely related to paracetamol metabolism: paracetamol, paracetamol sulfate, paracetamol glucuronide, cysteine paracetamol, *N*-acetylcysteine paracetamol, cysteine paracetamol glucuronide, 3-methoxy-paracetamol glucuronide, 3-methoxy-paracetamol sulfate and the benzothiazine compound; the latter originated from deacetylated paracetamol (Chen et al. 2008). The unknown metabolite with RT of 6.6 and *m*/*z* 449.045 was likely related to paracetamol detoxification, since its fragmentation spectrum showed a paracetamol

Table 4 Paracetamol metabolites, sulfur and nitrogen in urines collected over d14

	Concentration (mg/l)	Quantity in 24-h urines (mg/day)
Paracetamol	61.5±24.0	147±73
Paracetamol sulfate	351±48	650±86
Paracetamol glucuronide	1,975±360	3,584±653
Cysteine paracetamol	24.5±1.6	46.7±4.0
Total cysteine paracetamol [a]	127±17	228±25
N-acetylcysteine paracetamol	90.8±12.0	170±25
Total *N*-acetylcysteine paracetamol[a]	107±11	195±17
Sulfur in paracetamol metabolites	67.7±8.1	124±13
Total sulfur	342±41	624±64
Total nitrogen	6,793±743	12,413±1,218

Values are means±SEM

[a] Quantified after enzymatic hydrolysis as described in "Materials and methods"

Table 5 Urinary metabolites that are detected only at d14 (positive and negative ion modes)

RT	*m/z*	Identification
3.8	447.119	Cysteine paracetamol glucuronide[b]
4.2	328.103	Paracetamol glucuronide[a]
4.7	271.075	Cysteine paracetamol[a]
5.1	152.063	Paracetamol[a]
5.5	358.130	3-Methoxy-paracetamol glucuronide[b]
5.5	281.125	Benzothiazine compound[b]
5.9	248.031	Hydroxy-paracetamol sulfate[c]
6.3	232.031	Paracetamol sulfate[a]
6.6	449.045	Unknown[d]
7.7	313.091	*N*-acetylcysteine paracetamol[a]
9.3	198.063	$C_9H_{11}NO_4$[d]

RT retention time, *m/z* mass-to-charge ratio

[a] Identified compounds (identification with chemical reference standards)

[b] Putatively annotated compounds (e.g. without chemical reference standards, based upon physicochemical properties and/or spectral similarity with public/commercial spectral libraries)

[c] Putatively characterized compound classes (e.g. based upon characteristic physicochemical properties of a chemical class of compounds, or by spectral similarity to known compounds of a chemical class)

[d] Unknown compounds—although unidentified or unclassified these metabolites can still be differentiated and quantified based upon spectral data

residue. The unidentified metabolite with RT of 9.3 and *m/z* 198.063 was probably an endogenous metabolite exhibiting an undetectable level before treatment. The excretion of seven endogenous metabolites was increased with paracetamol treatment, whereas urinary levels of 15 endogenous metabolites were lower at d14 than before treatment (Table 6). Some of them contained sulfur: homocysteine and the unknown metabolite with RT of 6.9 and *m/z* 410.018 decreased with paracetamol treatment, whereas dimethyl sulfone and methionine sulfoxide increased. Unknown metabolites in Table 6 did not show any cysteine or *N*-acetyl-cysteine residues in their fragmentation spectra.

Discussion

Older persons are particularly exposed to the adverse effect of medication on the nutritional status (Chen et al. 1985; Pickering 2004). We have reported for the first time how independently living patients adjusted to a paracetamol curative treatment lasting 14 days. The excretion of paracetamol and more precisely of paracetamol sulfate was found lower than expected. Despite a large loss of sulfur in urinary paracetamol metabolites, morning blood glutathione, plasma sulfates and amino acids were not significantly affected by the treatment. Paracetamol treatment induced a spontaneous increase in dietary proteins leading to an increase in sulfur intake corresponding to half of paracetamol-induced sulfur loss. The large-scale metabolomic analysis indicates that long-term paracetamol treatment induced few metabolic perturbations, of which an increased oxidation of some sulfur-containing compounds.

Paracetamol metabolism in older persons

Recovery of paracetamol in 24-h urines at d14 was presently 77% of the daily dose. This value was lower than the 85% to 95% of recovery in the urine within 24 h observed in healthy adult subjects after a single therapeutic dose (Forrest et al. 1982). It is unlikely that absorption of paracetamol was affected in elderly, since it occurs by passive transport (Forrest et al. 1982). Since the therapeutic dosage was reported to be carefully applied, the low paracetamol recovery may be explained by an increased elimination through the bile, a decrease in renal excretion due to the aging-associated diminution in renal function (Zhou et al. 2008), or a prolonged retention in the body due to overwhelmed detoxification capacities. The latter point is supported by the low excretion of paracetamol sulfate, which represented less than 20% of paracetamol excreted in urine, whereas it usually amounted to 30% (Forrest et al. 1982; Critchley et al. 1986). A low partial metabolic clearance of paracetamol sulfate has already been reported in elderly (Miners et al. 1988). The lower excretion of paracetamol as sulfate conjugate was compensated by a higher excretion as glucuronide conjugate, which represented 65% of paracetamol excreted in urine, whereas it usually amounts 55% (Forrest et al. 1982; Critchley et al. 1986). The intense glucuronidation of paracetamol was associated with a decrease in the excretion of 16alpha, 17beta-estriol 17-beta-D-glucuronide (Table 6) suggesting a competition between paracetamol and endogenous steroid for this reaction. Contributions of *N*-acetylcysteine and cysteine

Table 6 Urinary metabolites that are modified by paracetamol treatment

RT	*m/z*	*P* value	Formula	Identification	Treatment effect
Positive ion mode					
0.6	424.904	0.038	?	Unknown[d]	–
0.7	136.049	0.012	$C_4H_9NO_2S$	Homocysteine[a]	–
0.9	142.087	0.040	$C_6H_{11}N_3O$	L-histidinol[b]	–
1.9	212.062	0.011	?	Unknown[d]	–
2.4	111.010	0.038	?	Unknown[d]	+
2.6	262.043	0.035	$C_9H_{12}NO_6P$	Phosphotyrosine[b]	–
3.0	367.162	0.036	?	Unknown[d]	–
3.7	95.016	0.055	$C_2H_6O_2S$	Dimethylsulfone[c]	+
4.3	224.009	0.016	?	Unknown[d]	+
4.5	151.060	0.003	$C_5H_{10}O_5$	Unknown[d]	–
4.6	166.043	0.001	$C_5H_{11}NO_3S$	Methioninesulfoxide[b]	+
7.4	262.043	1.4 E-05	$C_9H_{12}NO_6P$	4,6-Diamino-5-formamidopyrimidine[c]	+
8.8	326.121	0.016	$C_{11}H_{19}NO_{10}$	*N*-glycoloyl-neuraminate[c]	–
9.6	346.230	0.031	?	Unknown[d]	+
10	289.124	0.011	?	Unknown[d]	+
11.4	464.203	0.023	$C_{24}H_{31}O_9$	16alpha,17beta-estriol17-beta-D-glucuronide[b]	–
11.5	255.128	0.039	$C_{18}H_{22}O$	3-Deoxyestrone[b]	–
20.3	472.375	0.042	?	Unknown[d]	–
Negative ion mode					
6.9	178.047	0.001	$C_9H_9NO_3$	Hippurate[a]	–
6.9	410.018	0.020	$C_{13}H_{15}O_{13}S$	Unknown[d]	–
11.8	481.248	0.027	$C_{19}H_{38}N_4O_{10}$	Unknown[d]	–
13.2	465.240	0.004	?	Unknown[d]	–

RT retention time, *m/z* mass-to-charge ratio

[a] Identified compounds (identification with chemical reference standards)

[b] Putatively annotated compounds (e.g. without chemical reference standards, based upon physicochemical properties and/or spectral similarity with public/commercial spectral libraries)

[c] Putatively characterized compound classes (e.g. based upon characteristic physicochemical properties of a chemical class of compounds, or by spectral similarity to known compounds of a chemical class)

[d] Unknown compounds—although unidentified or unclassified these metabolites can still be differentiated and quantified based upon spectral data

conjugates to paracetamol excretion are in the range of published values (Forrest et al. 1982; Critchley et al. 1986). In elderly, phase II reactions (conjugation) seem to be more preserved than phase I reactions (Benedetti et al. 2007). Therefore, the low excretion of paracetamol as sulfate conjugate appears to be due to a low availability of the sulfate donor, 3′-phosphoadenosine 5′-phosphosulfate, whose precursor is inorganic sulfate issued from cysteine catabolism. The hepatic supply of 3′-phosphoadenosine 5′-phosphosulfate has already been suggested to be depleted with a single dose of 3 g compared to 0.5 g paracetamol (Slattery et al. 1987). Despite the fact that fasting plasma sulfates were not altered by paracetamol treatment (Table 3), a transient decrease in peripheral/tissular sulfates could have occurred. Indeed serum sulfates has been reported to decrease 2 h after administration of paracetamol to mice (Hazelton et al. 1986). The hepatic pool of sulfate and/or activities of hepatic 3′-phosphoadenosine 5′-phosphosulfate synthetic enzymes should have been the limiting factor(s). Unfortunately, the effect of aging on these activities is not documented.

Paracetamol treatment and sulfur-containing compounds

Based on the literature, diminutions in the peripheral concentrations in sulfur-containing compounds (GSH, sulfur amino acid, sulfate) were expected to be induced by the long-term treatment of paracetamol. In the present study, blood and plasma compounds were quantified in fasting state, i.e., about 14 h after evening meal and third daily ingestion of paracetamol. Meal and paracetamol post-ingestion kinetics would have been more powerful to evaluate these alterations. Kinetics measurements were not performed for ethical reasons: elderly subjects were suffering. In volunteers aged 18 to 40 years, baseline plasma cysteine, cystine, reduced and oxidized forms of GSH concentrations were unchanged after 2 day of paracetamol treatment at the dose of 1 g/day (Mannery et al. 2010). Nevertheless, in the latter study some differences appeared significant at some time-points across the 12-h kinetics. Therefore the lack of differences in baseline concentrations of peripheral sulfur-containing compounds does not mean that intensities of their metabolic pathways were not affected in the present study. These modifications are confirmed by the increase in the dietary intake of proteins, by the quantity of sulfur excreted into urines to support paracetamol detoxification and by modifications in endogenous sulfur-containing compounds in urine.

Nutritional adjustment to paracetamol treatment

The major adjustment occurring in older persons under long-term paracetamol treatment was an increase in dietary protein intake with maintenance of energy intake (Table 2). Before treatment, dietary protein intake was 0.93±0.08 g/kg/day protein, which is lower than values reported for French healthy persons of mean age 68 y (1.2 g/kg/day; Rousset et al. 2003) and for +70-year-old Americans (1.0 g/kg/day; Fulgoni 2008). This can be explained by their quite low-energy intake (23 kcal/kg/day), likely due to their low level of physical activity. The increase in dietary proteins up to 1.06±0.07 g/kg/day was mainly focused on animal proteins, which contain more sulfur amino acid than vegetable proteins do (Block and Weiss 1956). This increase did not happen for energetic purpose since energy intake was maintained by decreasing alcohol (Table 2) and carbohydrates (not shown) intakes. The increased intake in proteins, especially from animal sources, most likely occurred to satisfy the paracetamol-induced increase in sulfur amino acid demand. The extra-ingestion of sulfur amino acids was evaluated at 0.30 g/day, which corresponded to 72 mg/day sulfur. This amount equaled half of the sulfur excreted in urines as paracetamol conjugates. Considering that dietary proteins contain 35 mg of sulfur amino acid/g of protein (Patureau Mirand 2003), older persons treated with paracetamol consumed 37 mg sulfur amino acid/kg/day, which is much higher than the published population-safe intakes of 21–27 mg/kg/day (Di Buono et al. 2001; Kurpad et al. 2003). The increase in dietary proteins probably allowed saving whole body proteins. Indeed the urinary excretion of nitrogen converted to proteins (N×6.25) was not significantly different from the dietary protein intake. In addition, fasting amino acids were also unchanged and the large-scale metabolomic analysis did not reveal significant variation in 3-methylhistidine, a marker of proteolysis.

Metabolic effects of paracetamol treatment

Despite the adjustment to long-term paracetamol treatment through the increase in protein intake, the large-scale analysis of urinary metabolites revealed a decrease in homocysteine and hippurate supporting an increased endogenous synthesis of cysteine. The variation of hippurate, which is a metabolite of glycine, can be related to endogenous synthesis of cysteine. Indeed, cysteine synthesis requires serine which can be synthesized from glycine. Decreased hippurate has already been reported in urine from rats receiving paracetamol (Sun et al. 2008). The increased excretion of dimethyl sulfone and methionine sulfoxide meant that oxidation of sulfur-containing compounds occurred, likely as a consequence of decreased antioxidant capacities of cysteine and GSH. This is supported by the observation that paracetamol treatment oxidized the plasma cysteine/cystine redox potential in human (Mannery et al. 2010). An increased oxidative stress could have clinical effect, since oxidative stress has been implicated in the pathogenesis of cancer, diabetes mellitus, atherosclerosis, neurodegenerative diseases, rheumatoid arthritis, ischemia/reperfusion injury, obstructive

sleep apnea, other diseases, and also in senescence (Dröge 2002).

We speculate that whole body proteins would have been mobilized if older persons under paracetamol had not increased their sulfur amino acid ingestion. Assuming that skeletal muscle proteins would have been mobilized to provide 0.30 g/day sulfur amino acids for 14 days, the protein loss would have been issued from 470 g of skeletal muscle, based on 20% proteins in muscles and 4.5% sulfur amino acids in muscle proteins (Block and Weiss 1956). The amount of proteins that should be mobilized to detoxify 3 g/day paracetamol for 14 days can be estimated from the ratio between sulfur and nitrogen measured in urine. Daily sulfur contained in urinary paracetamol metabolites corresponded to 2.47 g/day nitrogen. This amount would necessitate the mobilization of 216 g of protein ($N \times 6.25$) over the period of treatment, corresponding to 1 kg of skeletal muscle. Post-operative patients treated with paracetamol, and receiving no nutrients for few days, would face similar situation. Long-term treatment with other drugs whose detoxification requires sulfate and glutathione detoxification might also led to such alterations.

Study limitations

There are several potential limitations to our study, which should be acknowledged. As we performed the experiment in independently living older patients, our results might be not applicable to other older persons, especially nursing home and long-term care residents and hospitalized patients. A lower liver volume and a lower urinary clearance of paracetamol glucuronide (single intravenous 0.5 g dose) have been reported in frail hospitalized elderly, compared to fit elderly or young subjects (Wynne et al. 1990). Nevertheless, no paracetamol accumulation appeared to occur in polymedicated very old patients (Bannwarth et al. 2001).The major problem for frail elderly would be their inability to adjust through an increase in dietary protein intake. Consequently, body proteins are expected to be mobilized to support paracetamol detoxification in this population. The accuracy of dietary evaluations depends on the quality of reporting made by patients and the contents of dietary composition database. Careful interviews were performed by a dietician in order to increase the accuracy in food intake evaluation at both time points. There is also a potential bias with respect to large-scale metabolomic approach due to the comparison of the second morning urine at the beginning of the treatment and the 24-h urine at the end. The lack of dietary standardization might have increased inter-individual variability and hide some tenuous variations for endogenous metabolites (Winnike et al. 2009). Nevertheless, a standardization of the diet would have introduced another bias regarding the spontaneous modification in animal protein intake. Paracetamol therapeutic dose was 3 g/day, which is in the line with French recommendation. Food and Drug Administration of USA (FDA) has asked for a limited daily dose of 2.6 g a day of paracetamol (see www.fda.gov/medwatch) while 4 g had been allowed in industrialized countries. This decision led to a controversy (Graham et al. 2010). Our results plead for a careful evaluation of paracetamol needs and individualized dosages depending on the clinical state of the patient, especially the elderly. However, paracetamol presents definitely less side effects than nonsteroidal anti-inflammatory drugs on a long-term basis and should be used for its valuable analgesic properties.

Conclusion

Despite a quite low excretion of paracetamol as sulfate conjugate in independently living older patients, the amount of sulfur needed to detoxify 3 g/day paracetamol equaled 20% of the sulfur provided by 1.06 g/kg/day dietary proteins. Their whole body protein stores were preserved at d14. Nevertheless, some metabolic deteriorations occurred (decreased elimination of endogenous steroids, enhanced oxidation of sulfur-containing compounds, and others non-identified modifications), whose physiological consequences are to be determined. Oxidation of sulfur-containing compounds was likely due to decreased anti-oxidative capacities of cysteine and glutathione induced by their high utilization for detoxification purpose. Older persons adjusted to long-term paracetamol treatment by increasing their dietary protein intake (mainly those from animal origin) confirming that usual consumption was insufficient. The sulfur amino acid daily intake was adjusted at 37 mg/kg/day, which is much higher than the corresponding published population-safe intakes. It appears that nutritional adjustments should be made

to compensate for the possible deficits induced by paracetamol consumption. The benefit/risk ratio of this drug might be largely increased by nutritional adaptations and guidelines.

Acknowledgments We thank all persons who collaborated in the study, especially Bertille Lamandé for clinical study setting-up, Nordine Hafnaoui for amino acid analysis, Jean-François Martin for statistical analysis of metabolomic raw data, Charlotte Joly for large-scale metabolomic analysis, Hélène Lafarge for literature acquisition and the volunteers who participated in the study. The research was supported by the Institut National de la Recherche Agronomique (INRA), France.

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