Supporting Information

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SI Text

Supplementary Methods. ***Outline of study protocol.*** The clinical phase of the study (study PRC-03–01) was performed over a number of different days in March and April 2003 at the Pfizer Research Centre at Canterbury, Kent, U.K. Ninety-nine nonsmoker men, aged between 18 and 64 years old on the day of their participation in the study, were recruited for an ethically approved 1-day clinical trial in which each participant was identified by a subject number ranging from 1 to 99. The study protocol did not specify a standard diet but placed certain restrictions on the diet and on alcohol consumption. Volunteers were only eligible if not taking drugs, herbal medicines, or dietary supplements. On the day of their participation in the study, the weight and height of each subject were recorded. The ethnicity of each subject was also recorded. Each subject provided a "snapshot" predose urine sample and then took two 500 mg tablets of acetaminophen (paracetamol BP) by mouth with 250 mL of water. After dosing, each subject was required to collect all of the urine that he produced over 2 consecutive time periods, namely 0–3 h and 3–6 h from dosing. Each subject was requested to empty his bladder as completely as possible at the end of each postdose time period and the mass of urine produced by each subject over each period was measured. Representative samples were stored frozen pending analysis at Imperial College London, London, U.K. with duplicate samples being sent for freezer storage at Pfizer, Sandwich, Kent, U.K.

Preparation of urine samples for ^{1}H NMR (nuclear magnetic resonance) spectroscopic analysis. The urine samples were prepared for NMR analysis by mixing 440 μL of urine with 220 μL of phosphate buffer [an 81:19 (vol/vol) mixture of 0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4, pH $\approx$7.4, to which 0.33% (wt/vol) sodium azide had been added to hinder bacterial proliferation]. The urine-buffer mixture was left to stand for at least 10 min at room temperature and then ultracentrifuged at 13,000 rpm for a further 10 min to remove suspended particulates. 550 μL of "clear" buffered urine was transferred to an appropriate sample vial and 55 μL of a TSP/D_2O solution added to give a final TSP concentration of 1 mM. TSP (sodium 3-trimethylsilyl-[2,2,3,3-$^2H_4$]-1-propionate) is a chemical shift reference compound (δ 0) used in the NMR experiment and the D_2O provided a field/frequency lock for the NMR spectrometer. The vials were capped and the samples frozen pending analysis. "Blank" samples were prepared at the same time and using the same solutions. The method of preparing the blanks was identical to that used for the urine samples except that the stabilized phosphate buffer was used instead of urine.

[Note that some workers acidify urine before ^{1}H NMR spectroscopic analysis. However, organic sulfates such as acetaminophen sulfate (S) and *p*-cresol sulfate (PCS) are expected to be liable to hydrolyze to form acetaminophen and *p*-cresol, respectively, and, in comparison to the rate at neutral pH, the rate of such hydrolysis is expected to be much increased at low pH. Thus, we would recommend use of the sample preparation procedure described above.]

^{1}H NMR spectroscopy and processing. The ^{1}H NMR spectra of the prepared urine samples and blanks were acquired at 600 MHz at a nominal 303 K on a Bruker AVANCE 600 NMR spectrometer equipped with a flow probe and operated by means of the Xwinnmr software (all from Bruker Biospin). The noesypr1d ("noesypresat") pulse sequence was used to acquire the data and to suppress the water signal during a relaxation delay of 3 s and during the pulse sequence mixing time of 0.1 s. Each spectrum was acquired using 8 dummy scans, 128 real scans, 32,768 time domain points, a 7,200-Hz spectral width, and an acquisition time of 2.3 s per scan. The spectral acquisitions were automated using the Iconnmr software and a BEST sample changer (both Bruker Biospin). The overall run time per sample was $\approx$20 min.

One-herz line-broadening (lb 1 Hz) was applied to the ^{1}H NMR spectra of the predose samples by means of an exponential multiplication of the free induction decay signal and these time-domain data were Fourier-transformed to frequency-domain spectra with a single zero-filling and with a digital filter (bc_mod qfil; bcfw 0.3 ppm) used to reduce the size of the residual water signal. The resulting spectra were manually phased to give an even baseline around the NMR signals and the baseline of each spectrum was manually adjusted to zero intensity using a straight-line baseline correction algorithm. The chemical shift scale was then set by assigning the value of δ 0 to the signal from the added reference compound (TSP). All these operations were performed on a Silicon Graphics computer using the Xwinnmr software (Bruker Biospin). The ^{1}H NMR spectra of the postdose samples were processed similarly to the above method (but with lb 0.3 Hz instead of lb 1 Hz and without using a digital filter) and subsequently with resolution enhancement (Gaussian multiplication, lb -1 Hz, gb 0.5, si 128 K) to better resolve the *N*-acetyl peaks from acetaminophen and its metabolites.

Quantitation of acetaminophen-related compounds excreted postdose. Conveniently, for the purposes of their urinary quantitation, acetaminophen and its principal metabolites each contain a common structural feature, an *N*-acetyl group that produces a single ^{1}H NMR peak in the vicinity of δ 2.1–2.2. Thus, with the right analytical conditions, these *N*-acetyl signals provide a basis for measuring the total number of moles of acetaminophen-related compounds excreted. Furthermore, because there is some intercompound variation in the exact chemical shift of the *N*-acetyl peak, the various signals also provide a basis for measuring the relative amounts of the different compounds (1). However, in the present work we found that not all of the different *N*-acetyl signals could be readily resolved despite the use of resolution enhancement. Thus, in our present study, we simply quantified the acetaminophen-related compounds as acetaminophen sulfate (S), acetaminophen glucuronide (G), and "other" with our final emphasis resting on determining the S/G ratio and the amounts of S and G excreted.

Although these data are not reported, the total amount of acetaminophen-related compounds excreted in the urine by each individual during each postdose collection was first estimated, by reference to the signal for the added TSP and the mass of urine collected, as $a \cdot c$, where a is [(the integral for the δ 2.210–2.135 region)/(TSP integral)], obtained from the non-resolution-enhanced spectrum, and c is the mass of urine collected. The use of this formula, which provides a relative measure of the total excretion of acetaminophen-related compounds, relies on the constancy of the NMR sample preparation [which was confirmed by repeated preparation and analysis of all of the postdose samples (see later and Tables S3 and S4)]. Additionally, the use, within the above formula, of urinary mass (c) in place of urinary volume relies on the different urine samples having very similar densities and this assumption was supported by density measurements on a number of representative 0–3 h samples, which indicated that urinary density varied within $\pm$ 2% of the average value. The accuracy of the estimate obtained from the given formula also relies on the different N-acetyls having

similar T1 values and on there being no significant contributions from other compounds. Assuming these conditions are met, the measure provided by this formula should be proportional to the number of moles of acetaminophen-related compounds excreted.

The second stage in the analysis was to use the resolution-enhanced versions of the postdose spectra to determine the proportions of the total δ 2.210–2.135 integral derived from S, G and "other." Thus, for each resolution-enhanced spectrum, after integrating the whole of the δ 2.210–2.135 region (which automatically assigned the total integral a value of 1) the individual fractional contributions from the S and G peaks [at ≈δ 2.182 (S) and at ≈δ 2.166 (G), respectively] were determined by further integration and recorded to 2 decimal places. The fractional "other" contribution was subsequently calculated by subtracting the sum of the S and G fractions from 1. The S/G ratio was calculated from the ratio of the S and G fractions. [Our further NMR analysis, under conditions of full relaxation between acquisition pulses, indicates that these S/G ratios will be close approximations to the relevant mole ratios].

For each subject and postdose collection period, measures of the individual amounts of S and G excreted were also determined. Thus, for example, the amount of S excreted was calculated as a · b · c where: a is [(the integral of the δ 2.210–2.135 region)/(TSP integral)], obtained from the non-resolution-enhanced spectrum; b is the fraction of the δ 2.210–2.135 integral that arises from S, determined from the resolution-enhanced spectrum; c is the mass of urine collected; and the use of this formula relies, as before, on the constancy of the sample preparation and on the density of the postdose urine samples being nearly constant.

As a check on the preceding analysis, the S/G ratio determinations were also performed using direct integration of the relevant resolution-enhanced peaks and the integrations recorded without rounding. The results obtained were compared with the original data (see Tables S3 and S4). As a further check on the above determinations, further measures of S/G, amount of S excreted, and amount of G excreted were obtained from the non-resolution-enhanced versions of the spectra, using the integrals of selected aromatic signals [the multiplets centered at ≈δ 7.32 (from S) and at ≈δ 7.15 (from G)], and the data obtained compared with the data derived using resolution enhancement (see Tables S3 and S4). Thus, for example, a measure of amount S excreted was determined as [(integral of δ 7.32 multiplet)/(TSP integral)] · [mass of urine collected]. Again, the use of such a formula depends on the constancy of the sample preparation method and on the near constancy of the urinary density.

The accuracy of the analyses described above depends on the absence of significant spectral overlaps from other compounds and the potential for such overlaps was expected to be lower when the determinations were based on the resolution-enhanced *N*-acetyl peaks rather than when the determinations were based on the aromatic signals. Thus, it is the N-acetyls-based data that are reported in the paper. However, reasonably good correlation was achieved between the 2 sets of data (see Tables S3 and S4), which strongly supports the validity of the reported data since any overlapping compounds would be unlikely to affect the measured *N*-acetyl and aromatic signals to the same extent. Additional confidence was generated by visual inspection of the shapes of the measured signals, by the manual placement of integration limits, and by the use of a high field spectrometer (operating at 600 MHz for ^{1}H), which increases signal dispersion compared with work at lower field strengths. No correction was made for any predose signals occurring in the measured regions, although the subsequent statistical analysis of the *N*-acetyls-derived data was performed both with and without 15 subjects who were found by UPLC-MS (see later discussion) to have acetaminophen metabolites in their predose urine samples. However, it is noteworthy that, of these 15 subjects, only 3 had sufficient levels of acetaminophen metabolites in their predose samples that they were independently identified as such from the ^{1}H NMR analysis, which is considerably less sensitive than the UPLC-MS analysis. In considering potential peak overlaps, it should be recognized that, because of possible diurnal and acetaminophen-induced changes to the endogenous metabolites profile, the predose spectra are not necessarily fully representative of the postdose "background" on which the spectra of acetaminophen and its metabolites are superimposed. However, the predose spectra do provide some indication of the potential for distortion of the measured quantities (S excretion, G excretion, and S/G ratio) by preexisting urinary components. Thus, after normalization to constant creatinine, the *N*-acetyl region of each postdose spectrum was compared with the same region in the corresponding predose spectrum. Some similar comparisons were also performed in relation to the aromatic multiplets from S and G located at ≈δ 7.32 and at ≈δ 7.15, respectively. In principle, because the average S/G ratio was less than 1, the measurements of S would be expected to be more vulnerable to overlaps than the measurements of G, with the lower S/G ratios in the 3–6 h collection increasing that vulnerability. However, the comparison of individual predose and postdose spectra revealed that any predose overlaps tended to be relatively insignificant. Furthermore, because of the predose to postdose association indicated by this study, we looked in particular to see if any of the peaks from *p*-cresol sulfate (PCS) and phenylacetylglutamine (PAG) could have had an impact on our S and G determinations and found that they could not. We also considered the possibility that, by competing with *p*-cresol for sulfonation, the dosed acetaminophen might have caused an increase in the urinary excretion of either *p*-cresol or *p*-cresol glucuronide. However, there is no possibility that the peaks arising from these 2 compounds could have distorted the *N*-acetyls-based analysis of the acetaminophen-related compounds excreted postdose. Thus, we found by analysis of authentic *p*-cresol (Aldrich C85751) that its methyl group resonates at ≈δ 2.26 while the literature (2) indicates that the methyl group of *p*-cresol glucuronide resonates at ≈δ 2.31 [which is entirely consistent with our expectation, by analogy with the situation for acetaminophen and its metabolites, that its chemical shift would be intermediate between the corresponding peaks for *p*-cresol sulfate (at ≈δ 2.35) and *p*-cresol (at ≈δ 2.26)].

The validity of the analytical approach used is further supported by the earlier work of Bales et al. (1), who had also used ^{1}H NMR spectroscopy to study the urinary excretion of acetaminophen and its metabolites and who had recorded, for reference purposes, the ^{1}H NMR spectra of 9 known or potential acetaminophen metabolites. They too had used the *N*-acetyl signals as the basis for the urinary excretion measurements and they found that their results agreed broadly with those reported by other workers using other methods (on other samples).

As reported in Table S1, we found that the S/G ratio and the amounts of S and G excreted did not correlate with subject body mass. Consequently, to avoid producing postdose parameters that were correlated with body mass (and, thereby, to avoid the possibility of building a model or models that effectively only predicted body mass), our analysis was mainly based on using these postdose parameters without correction to unit body mass. (In any case, it would also have been questionable whether a body mass correction was appropriate for the S/G ratio). However, we also found that correcting the various parameters (S/G ratio, amount S excreted, and amount G excreted) to unit body mass made very little difference to the graphical relationships between these parameters and the predose PCS/creatinine ratio.

Quantitation of* p*-cresol sulfate (PCS) excreted predose. It was not possible to directly measure predose PCS excretion because of the "snapshot" nature of the predose sample collection. Con-

sequently, the predose samples did not represent excretion over a measured period. Furthermore, it was not regarded as worthwhile to measure only a urinary PCS concentration because such concentrations would be expected to be markedly affected by intersubject differences in fluid consumption. Consequently, PCS was quantified relative to creatinine using the ^{1}H NMR spectra of the predose urine samples, which had all been acquired and processed in an identical manner. Creatinine is generally accepted as an internal reference compound for urinary metabolite quantitation (3) but the amount of creatinine excreted in the urine is known to be affected by gender and ethnicity (4). However, in the present study, all of the subjects were male and all but one was reported as "white" (see *Supplementary Data*). Thus, the approach taken was deemed appropriate. Further information on the factors affecting urinary creatinine excretion, which is stated to depend primarily on muscle mass, is provided in the Geigy Scientific Tables (5). These factors include subject age and the meat content of the diet. In principle, for 2 otherwise-identical individuals of different overall size, we would expect, by simple scaling, to see different amounts of PCS and creatinine excreted but identical PCS/creatinine ratios. Thus, to a first approximation, PCS/creatinine ratios would be expected to be independent of body mass and this was supported by our own findings (see Table S1). Another advantage of measuring PCS relative to creatinine, rather than relative to the total area of peaks in the NMR spectrum, was that this provides a parameter that is open to future investigation using conventional analytical methodologies. For this quantitation, the PCS methyl signal at $\approx\delta$ 2.348 was chosen in preference to the PCS aromatic signals centered at $\approx\delta$ 7.210 and δ 7.285 because of its greater peak height (and hence greater signal to noise ratio) and because the aromatic signals appeared to be more prone to overlap by peaks from other compounds. [However, using creatinine-normalized spectra, we were reassured to find an excellent linear relationship (correlation coefficient 0.985) between the spectral intensity at δ 7.219 (corresponding to a point on 1 of the aromatic peaks of PCS that appeared to be less prone to overlap) and the spectral intensity at δ 2.348. This provides strong support for using the δ 2.348 signal to quantify PCS]. For the quantitation, the creatinine methylene at $\approx\delta$ 4.06 was chosen in preference to the creatinine methyl at $\approx\delta$ 3.05 because, at a near neutral pH, it is known that the latter signal can be overlapped by the methyl signal of creatine, if creatine is present.

Initially, the integral ratio of the chosen peaks, at $\approx\delta$ 2.348 and at $\approx\delta$ 4.06, respectively, was obtained using MATLAB, after applying an overall straight-line baseline correction to each spectrum in Xwinnmr. Subsequently, using Xwinnmr, we set out to obtain a more accurate measurement of the PCS/creatinine integral ratio for each spectrum with local baseline corrections applied to the peaks of interest before manual integration. The integral ratios (I.R.) obtained from that procedure provide an estimate of the relative amounts of PCS and creatinine present, with the accuracy of this estimate depending on the absence of significant peak overlaps and on correct positioning of the local baseline corrections. The integral ratios obtained are not intended to be mole ratios but are relative measures that will be affected by the different number of protons giving rise to the 2 signals (PCS CH_3 vs. creatinine CH_2), by any relaxation differences between the 2 signals (because the NMR experiment was not designed to allow full relaxation between the multiple acquisition pulses) and by the slight suppression of the creatinine CH_2 signal by the digital filter that was applied to reduce the magnitude of the residual water peaks. Further partial suppression of the creatinine CH_2 signal could potentially arise through proton exchange with water with such exchange requiring that the creatinine methylene takes part in keto-enol tautomerism with its adjacent carbonyl group. The integral ratios obtained might potentially also be affected by the 1 Hz line broadening that was applied to increase signal to noise. However, all of the experimentally controllable factors affecting integral ratios were constant and unchanging for our experiment and for the purposes of our analyses can be ignored. Likewise, the noncontrollable factors are expected to be either constant or very nearly constant.

The percentage error associated with these integral ratio measurements is expected to be greater when the level of PCS is low. In this situation, overlaps from other compounds are potentially more significant and it becomes more difficult to determine the appropriate baseline correction and the limits of the integration. However, in the present work, accurate quantitation of low level PCS was not so important and it was more important to be confident of the quantitation when the PCS level was high. As a precaution, we looked specifically to see if the measured predose PCS/creatinine integral ratios (IR) could have been affected by the possible presence of particular compounds (acetaminophen, acetaminophen sulfate, acetaminophen glucuronide, *p*-cresol, and phenylacetylglutamine) and found that they could not. Furthermore, we do not envisage there having been any significant distortion of the measured predose PCS/creatinine integral ratios by *p*-cresol glucuronide. As a further precaution, we also examined the constancy of the ratio of (integral creatinine CH_3)/(integral creatinine CH_2) obtained in the ^{1}H NMR spectra of the predose samples. Ignoring 2 spectra where there was obvious overlap of the creatinine CH_3 signal by some other compound, the measured ratio was found to vary between $\approx$1.6 and 1.9 with most values falling between 1.6 and 1.8, with no sign of any run order-related change.

Identification of relevant compounds. *1. Acetaminophen.* The NMR signals from this compound were identified by comparison of ^{1}H NMR spectra of postdose urine before and after addition of authentic acetaminophen (Sigma-Aldrich A5000).

2. Acetaminophen sulfate (S). The NMR signals from this metabolite were identified by comparison of ^{1}H NMR spectra of postdose urine before and after addition of authentic acetaminophen sulfate (Sigma-Aldrich UC448).

3. Acetaminophen glucuronide (G). The NMR signals from this metabolite were identified by comparison of ^{1}H NMR spectra of postdose urine before and after addition of authentic acetaminophen glucuronide (Sigma-Aldrich A4438).

4. Phenylacetylglutamine (PAG). The NMR signals from this metabolite were identified by comparison of ^{1}H NMR spectra of predose urine before and after addition of authentic phenylacetylglutamine (LGC certified reference material 169.01).

5. p-cresol sulfate (PCS). The ^{1}H NMR signals centered at $\approx\delta$ 7.210 and at $\approx\delta$ 7.285 showed the typical pattern for a para-disubstituted aromatic compound and STOCSY analysis (6) was used to identify ^{1}H NMR signals originating from the same molecule. This indicated that the molecule might be *p*-cresol or *p*-cresol sulfate and that it was very unlikely to be *p*-cresol glucuronide. Comparison of ^{1}H NMR spectra of predose urine recorded before and after addition of authentic *p*-cresol (Sigma-Aldrich C85751) proved that the unknown molecule was not *p*-cresol. Changes in the ^{1}H NMR spectra of predose urine samples treated with sulfatase (Sigma-Aldrich S9626) were consistent with the molecule being *p*-cresol sulfate, although the sulfatase used was stated to also have some glucuronidase activity. In these experiments using sulfatase, and in similar experiments using acid hydrolysis instead of sulfatase, the peaks assigned to PCS decreased in intensity and *p*-cresol was produced. The ^{1}H NMR signals from this metabolite (PCS) were finally identified by comparison of ^{1}H NMR spectra of predose urine before and after addition of authentic *p*-cresol sulfate (synthesized, purified, and characterized in-house). Yet further confirmation was subsequently obtained using a second lot of

PCS that had been synthesized and characterized in-house without purification.

6. Creatinine. The NMR signals from this metabolite were identified by comparison of ^{1}H NMR spectra of predose urine before and after addition of authentic creatinine (Sigma-Aldrich C4255).

Synthesis, purification, and characterization of p-cresol sulfate (PCS). *p*-Cresol sulfate (PCS) was synthesized by the slow addition of chlorosulfonic acid (Fluka 26388, purity ≥ 98.0%) to an ice-cooled solution of *p*-cresol (Sigma-Aldrich C85751, purity 99%) in pyridine (Sigma-Aldrich 270407, purity ≥ 99.9%) (7). After removing volatiles with a rotary evaporator, column chromatography (silica, acetonitrile/methanol) was used to isolate a purified PCS fraction from the remainder of the reaction mixture. After dissolution in phosphate buffer, the identity of the isolated PCS was established by ^{1}H NMR spectroscopy and by UPLC-MS [ultra performance liquid chromatography with detection of eluted compounds by mass spectroscopy; −ve ion mode; *m/z* 187 (molecular ion) and *m/z* 107 (corresponding to loss of SO_3)] with further ^{1}H NMR analysis confirming that the material identified as PCS was not an impurity of the *p*-cresol used in its synthesis. A second lot of PCS was synthesized similarly but with the amount of added chlorosulfonic acid adjusted to give nearly complete conversion of *p*-cresol to PCS. This second lot of PCS was characterized by ^{1}H NMR spectroscopy and by UPLC-MS without purification.

Further analysis of postdose urine samples. In 2007, ≈4 years after the original NMR analysis, the postdose urine samples (which had been stored during the interim period at −40 °C) were prepared for a repeat NMR analysis. The mode of preparation, using phosphate buffer (without sodium azide) and a solution of TSP in D_2O, was essentially the same as that used previously but on this occasion the samples were prepared in standard 5 mm NMR tubes rather than in vials and the final sample volume was 550 μL rather than 605 μL. ^{1}H NMR spectra were acquired using Xwinnmr and the acquisitions automated using Iconnmr and a standard "BACS" 60 position sample changer (all Bruker Biospin). The acquisition parameters were the same as used previously except that the zgpr pulse program was used instead of the noesypr1d program and, to save time, 64 scans were acquired per sample instead of the previous 128. After the normal processing, an S/G ratio was determined for each spectrum by integration of the relevant aromatic signal clusters at ≈δ 7.32 (S) and at ≈δ 7.15 (G) after manual application of local baseline corrections. The same signals were also used in obtaining measures of the concentrations of S and G in each individual urine sample. Thus, for each sample, values were determined for [(integral δ 7.32 cluster)/(TSP integral)] and [(integral δ 7.15 cluster)/(TSP integral)] and the results obtained were then compared with the equivalent data derived from the original 2003 spectra (see Tables S3 and S4). These comparisons provided a check on the reliability of the original sample preparation and also, thereby, on the reliability of the S and G excretion measurements derived from the original 2003 spectra.

Further checks were also performed to investigate how certain spectral parameters changed when the original noesypr1d NMR experiment was replaced by the simpler zgpr experiment with full relaxation between acquisition pulses (recycle time >20 s). Additionally, selected samples, having particularly distinctive spectral characteristics, were run manually to confirm that the NMR automation runs had maintained the correct sample order.

In 2008, 5 years after the original NMR analysis, the freezer-stored 3–6 h samples were prepared for analysis on a UPLC-MS system, which was also equipped with a diode array detector. The peaks arising from acetaminophen sulfate and acetaminophen glucuronide were confirmed from their characteristic mass spectra and were found to be well resolved from one another. For each sample, single wavelength (254 nm) data were extracted from the diode array results and used to calculate an acetaminophen sulfate/acetaminophen glucuronide ratio (S/G) for each sample. These ratios, which were independently determined, were found to correlate extremely well (correlation coefficient 0.991) with the S/G ratios derived from the original NMR analysis (2003; resolution-enhanced acetyls) of the 3–6 h urine samples. No outliers were found. This independent analysis fully supports the original NMR-based approach to measuring the S/G ratio in the postdose samples.

Further analysis of predose urine samples. In 2007, ≈4 years after the original NMR analysis, the predose urine samples (which had been stored at −40 °C) were prepared for a repeat NMR analysis. The mode of preparation, using phosphate buffer (without sodium azide) and a solution of TSP in D_2O, was essentially the same as that used previously but on this occasion the samples were prepared in standard 5 mm NMR tubes rather than in vials and the final sample volume was 550 μL rather than 605 μL. ^{1}H NMR spectra were acquired at 600 MHz using Xwinnmr and the acquisitions automated using Iconnmr and a standard "BACS" 60 position sample changer (all Bruker Biospin). The spectra were processed in Xwinnmr without the use of a digital filter and for each sample the PCS/creatinine ratio was determined by manual integration of the peaks at ≈δ 2.348 and ≈δ 4.06 after local baseline correction. The measured ratios were then compared with the PCS/creatinine ratios obtained from the original (2003) spectra (see Table S2). To support the subsequent UPLC-MS analysis (see later discussion) a hippurate/creatinine integral ratio was similarly obtained from each spectrum using the hippurate peak cluster at ≈δ 7.56 and the creatinine CH_2 peak at ≈δ 4.06. Additionally, selected samples, having particularly distinctive spectral characteristics, were analyzed manually to check that the NMR automation runs had maintained the correct sample order.

The 4-year-old predose urine samples were also analyzed by UPLC-MS (−ve ion mode) with appropriate standards (of S, G, PCS, creatinine, and hippurate) used to establish retention times and to optimize the chromatography. The original aims of the UPLC-MS analysis were (*i*) to get an independent measure of the PCS/creatinine ratios, and (*ii*) to identify subjects who had taken acetaminophen just before the start of the study by measuring the ratios S/creatinine and G/creatinine. However, creatinine was found to be poorly retained and barely detectable. Thus, the UPLC-MS method was redesigned so as to use hippurate as the internal reference point for quantitation and the "x"/hippurate values (where x is S, G, or PCS) obtained from UPLC-MS were multiplied by the relevant hippurate/creatinine conversion factors obtained from the associated NMR analysis. The PCS/creatinine ratios derived from a combination of UPLC-MS and NMR analysis were then compared with those derived solely from NMR (see Table S2) to check the validity of the NMR-based quantitation of PCS/creatinine. The S/creatinine and G/creatinine values obtained were plotted against subject number and 9 subjects with high values for both parameters were immediately identified as likely to have taken acetaminophen just before the start of the study, thereby being noncompliant with the study protocol. (Reassuringly, the 3 predose samples that showed the highest values for S/creatinine and G/creatinine by UPLC-MS had likewise been identified from the original NMR analysis as containing the highest levels of these metabolites). After excluding the 9 subjects with the highest predose values for S/creatinine and G/creatinine, the data for the remaining 90 subjects were reexamined and 6 further subjects were identified for exclusion on the basis of having predose G/creatinine values greater than the mean + 2 standard deviations. In our view, the total proportion of subjects (15/99) identified for exclusion on this basis was probably somewhat higher than it needed to be. However, our preference for the second stage of

the univariate statistical analysis (see later discussion) was to exclude too many subjects rather than too few.

In 2008, ^{1}H NMR analysis of selected samples indicated that the PCS methyl signal would not be overlapped by the signal that would arise from pyruvate. Further NMR analysis, at 400 MHz, showed that the integral ratio between the PCS methyl and the creatinine methylene was virtually unchanged when the recycle time between acquisition pulses was changed from 5.3 s to 21.3 s (zgpr pulse program). This finding supports the reliability of the abstracted PCS/creatinine ratios.

MATLAB procedures relating to Fig. 2. The δ 10 to -1 region of each processed and baseline-corrected predose spectrum (original data) was loaded into MATLAB, using the in-house MetaSpectra routine provided by O. Cloarec (formerly Imperial College London; now of Royal Holloway, University of London), wherein the data point interval was adjusted to 0.0002 ppm. The individual spectra were then normalized to a constant integral for the δ 4.05 to 4.07 region, which encompasses the creatinine methylene singlet, and assigned to 1 of 3 color-coded groups according to the magnitude of their S/G 0–3 h values. Group average spectra were calculated and color-coded plots of selected regions of the individual and group average spectra were prepared. The mode of plot preparation was to add, sequentially, the plots for the different groups and it should be noted that this could result in some obscuration of previously plotted spectra, as is clear from a comparison of Figs. 2 *A* and *B*.

Principal components analysis (PCA). PCA is a pattern recognition method that is used to look for inherent similarities between objects or samples for which multivariate data have been obtained. A major strength of this method is that it is "unsupervised" (i.e., it is not directed by knowledge of some external factor or factors). In the present work, PCA was carried out on the original (2003) predose ^{1}H NMR spectra (all subjects included) after segmentation into consecutive 0.04 ppm-wide segments and integration across each segment. During the segmentation process, which was performed using the AMIX software (Bruker Biospin), the integrals obtained were normalized to a constant value for the δ 4.07–4.05 region, which principally contains the creatinine methylene singlet. PCA was then performed (using the Pirouette 3.11 software from Infometrix) on the data for the δ 9.1–6.9 spectral region using mean-centered variable scaling and with the maximum number of extracted factors limited to 10. In examining the results of the PCA, the 99 subjects were assigned to 3 classes according to their individual S/G (0–3 h) values. Thus, the 25 subjects with the greatest S/G (0–3 h) values were assigned to class 1 and the 25 subjects with the lowest S/G (0–3 h) values were assigned to class 3. The remaining 49 subjects were assigned to class 2. The MATLAB function "ranksum" from Statistics Toolbox was then applied to the scores for each PC to determine if there was any significant separation of the class 1 and class 3 subjects. This test is equivalent to a Mann–Whitney *U* test. The appropriate Bonferroni correction was taken as 30 (see next section). Selected scores plots were also plotted for visual examination with the following color-coding of the subjects: class 1, blue; class 2, green; class 3, red.

(The above analysis was repeated after normalization of the predose spectral data to a constant value for the δ 3.07–3.03 region instead of to a constant value for the δ 4.07–4.05 region. The δ 3.07–3.03 region principally contains the creatinine methyl singlet but may also include a contribution from creatine).

Magnitude of Bonferroni correction. The Bonferroni correction was used to control potential false positives arising from the multiple hypothesis testing that typically occurs in metabonomic analyses. However, the exact magnitude of the Bonferroni correction(s) that should be used required careful consideration with different corrections being applied depending on the nature of the analysis performed.

Bonferroni correction for computational pattern recognition analyses. The rank of the predose data matrix ($\leq$ 99 when all subjects were included) sets an upper limit on the number of factors that could potentially be extracted using computational multivariate pattern recognition methods such as PCA and PLS (projection to latent structure). However, in the PCA performed in this study, the appropriate Bonferroni correction was taken as 30, on the basis that our typical approach to the PCA of a data set would involve three separate analyses (autoscaling, mean-centered scaling, pareto scaling) with a maximum of 10 factors permitted to be extracted in each PCA.

Bonferroni correction for our other analyses. These other analyses were based on sequentially comparing intergroup differences in the levels of each NMR-detectable predose sample component. For these analyses, the relevant factor was the number of NMR-quantifiable urinary components and this number is likely to change to some extent depending on the nature of the NMR equipment and experiment used. Although a figure of $>$200 ^{1}H NMR-visible metabolites per urine sample has been indicated by one source (8), supporting evidence was not provided and, from our own experience, our view is that this is likely to be a considerable overestimate when using, as in the present study, conventional (noncryo) NMR probes and typical current spectrometers operating at up to 600 MHz. Furthermore, even if such a number of metabolites were visible, the number of components that would be readily quantifiable without using spectral deconvolution methods and reference spectra would be considerably less because of baseline intensity variation, noise, and peak overlaps. Instead, we consider that a much more realistic estimate of the number of metabolites that are readily quantifiable by ^{1}H NMR spectroscopy is provided by the April 2006 Chenomx document titled "Targeted Profiling of Common Metabolites in Urine" by C. Vitols and H. Fu, which lists 80 metabolites stated as being commonly seen in the NMR spectra of human urine (www.chenomx.com). Furthermore, even if the number of readily NMR-quantifiable urinary metabolites was as high as 200, which we doubt, use of a Bonferroni correction of 100 has been deemed acceptable in such circumstances (9) because of the assumptions associated with the Bonferroni correction. In fact, if we follow a similar argument and have, as we suspect, less than 100 components in human urine that are readily quantifiable by ^{1}H NMR spectroscopy, then a Bonferroni correction of 50 might be considered adequate. However, in this work, we have applied a correction of 100 to err on the side of caution.

Numerical single variable discovery procedures. Methods such as PCA and PLS are aimed at simplifying the analysis of multivariate data where different variables may provide similar information. PCA is an unsupervised method aimed at finding inherent patterns in the data and is not directed by knowledge of external factors. PLS is a supervised method that is focused on finding patterns in the data that correlate with an external variable. However, these methods are potentially less suited to the discovery of single variables that may correlate with an external variable. To determine if any individual components of the predose spectra correlated with the variation in the S/G ratio postdose, we applied variable discovery procedures described in international patent application WO2004038602A1 (10). Thus, the creatinine-normalized predose spectra for the subjects at the 2 extremes of the S/G 0–3 h distribution were compared (using 25 subjects from each end of that distribution) and all of the integrated predose spectral bands were then ordered according to their ability to discriminate these two S/G classes. In this case, after applying the relevant Bonferroni correction (100) to the most significant spectral bands, only three bands were found to be significant at the 95% level of confidence. These bands (at δ 2.347, 7.213, and 7.278) correspond to the spectral features of PCS. The procedure was repeated for the S/G 3–6 h distribution

and the same bands were again the only ones found to be significant.

Univariate statistical analysis and exclusion criteria. For the key parts of the univariate statistical analyses, the approach taken was to perform the analysis (*i*) with all 99 subjects included and (*ii*) after excluding all those subjects who did not comply with, or who did not appear to comply with, the study protocol. It was not considered practical to screen the subjects for prior use of drugs other than acetaminophen and the specific reasons adopted for subject exclusion were: (*i*) incorrect sample collection; (*ii*) presence of ethanol in predose sample indicating recent alcohol consumption; (*iii*) presence of acetaminophen metabolites in predose sample indicating recent use of acetaminophen; and (*iv*) presence of ketone bodies in predose sample indicating that the subject was not eating normally. This led to the exclusion of 21 subjects, with some subjects being excluded for more than 1 reason, which indicates the difficulty of performing studies in human subjects. Statistical significance was assessed using the Mann-Whitney *U* test with these analyses being performed in SPSS 14 (SPSS) R 2.5.0 and R 2.5.1 [R Development Core Team (2007) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3–900051-07–0, URL http://www.R-project.org]. This analysis was provided by the Statistical Advisory Service of Imperial College London. However, the preceding statements do not apply to the statistical analysis of the PCA scores, which has been described in a previous section.

Supplementary Data. ***General note to supplementary data.*** The NMR-derived data reported in the paper are based on the original (2003) spectra with the postdose data being derived using the resolution-enhancement approach. Unless otherwise specified, it is these data that are referred to here. However, comparisons with results obtained by different methods and comparisons with later NMR data are also provided.

Ethnicity of subjects. Ninety-eight of the 99 subjects were described as "white." The remaining subject was described as "white/Mexican."

Comparison of acetaminophen metabolite data for the 2 postdose urine collections. One gram of acetaminophen is ≈0.0066 mol (formula weight of acetaminophen = 151.17). The average percentages of this dose recovered (as any acetaminophen-related compound) in the 0–3 h and 3–6 h urine collections were estimated as 27.9 and 27.4, respectively (i.e., ≈0.0018 mol was recovered in each 3 h collection). Furthermore, taken together, acetaminophen sulfate (S) and acetaminophen glucuronide (G) typically accounted for ≈85% of the total number of moles of acetaminophen-related compounds recovered in each postdose collection.

The average S/G ratios for the 2 postdose collections were found to be 0.711 (0–3 h) and 0.532 (3–6 h), respectively, with the S/G value for each individual subject always being lower in the 3–6 h collection than in the 0–3 h collection. Furthermore, there was a clear correlation between the S/G 0–3 h and S/G 3–6 h data ($r = 0.927$). However, a plot of these data revealed that 1 subject did not fit the normal correlation and it was judged, from this and from other indications, that this subject had not fully collected his 0–3 h sample. With this subject excluded, the correlation between S/G 0–3 h and S/G 3–6 h was slightly improved ($r = 0.948$).

The decrease in S/G ratios between the 0–3 and 3–6 h collections suggests that the 1-g acetaminophen dose is sufficient to cause a temporary decrease in sulfonation capacity. However, the average percentages of the 1 g acetaminophen dose recovered in the 2 postdose urine collections were estimated to be very similar (see previous note) and the data suggest that, in the 3–6 h collection, increased glucuronidation largely compensates for decreased sulfonation.

Interrelationships between different acetaminophen metabolite parameters within an individual collection. Having excluded the 0–3 h data for the 1 subject who appeared not to have collected his 0–3 h urine properly, the correlation coefficients between the observed S/G ratios and the amount of S excreted were found to be 0.510 (0–3 h) and 0.835 (3–6 h), respectively. Likewise, the correlation coefficients between S/G and the amount of G excreted were found to be −0.738 (0–3 h) and −0.803 (3–6 h), respectively.

As described elsewhere, our NMR-based analysis was almost entirely focused on the 2 principal urinary metabolites (S and G). However, a preliminary further analysis of the NMR spectra of selected 3–6 h urine samples was also conducted to determine if the proportion of acetaminophen excreted as the mercapturic acid showed any relationship to the S/G ratio. Thus, for 15 spectra from each end of the S/G distribution, the mercapturic acid's additional *N*-acetyl signal at ≈δ 1.86 was used to determine the amount of that compound as a proportion of the total amount of acetaminophen-related compounds excreted. Although there was some overlap in the results obtained for the 2 groups, this analysis indicated that the proportion found as the mercapturic acid was somewhat higher on average for the high S/G group than for the low S/G group (3.7% vs. 2.5%). This suggests that a low capacity for acetaminophen sulfonation might also be associated with decreased availability of hepatic glutathione, which would have potential consequences for the detoxification of reactive metabolites by glutathione conjugation and for combating oxidative stress. Further work will be required to thoroughly investigate this interesting possibility.

Relationships between the measured urinary parameters and age, body mass, and order of sample analysis. See Table S1.

Calculations and considerations regarding the potential significance of p-cresol sulfonation in regard to the amount of acetaminophen sulfate produced. In the 0–3 h collection, the average number of moles of acetaminophen sulfate (S) recovered was found to be 0.00062 and, excluding the 1 subject where there was strong evidence of incomplete sample collection, the range of values was found to be 0.00031–0.00087 mol S. Thus, for this collection, and with the 1 subject excluded, the difference between the 2 extremes was 0.00056 mol of S. More importantly, and still working with the 1 exclusion, the 25 subjects showing the highest S/G values were found to have excreted on average 0.00073 mol of S, whereas the 25 subjects showing the lowest S/G values were found to have excreted on average 0.00054 mol of S. The difference between these values is 0.00019 mol of S.

In the 3–6 h collection, the average number of moles of S recovered was found to be 0.00051 and the range of values was found to be 0.00025–0.00079 mol of S. Thus, for this collection, the difference between the 2 extremes was 0.00054 mol of S. Furthermore, the 25 subjects showing the highest S/G values were found to have excreted on average 0.00065 mol of S, whereas the 25 subjects showing the lowest S/G values were found to have excreted on average 0.00039 mol of S. The difference between these values is 0.00026 mol of S.

In our original ^{1}H NMR spectra of the predose urine samples, the maximum value for (integral PCS methyl peak)/(integral creatinine methylene peak) was found to be 0.182 and the ratio of these 2 peaks was subsequently found to be virtually unchanged when full relaxation was allowed between NMR acquisition pulses. Thus, the maximum observed predose PCS/creatinine mole ratio was ≈0.12 (since $0.182 \times \frac{2}{3} = 0.121$).

From the Geigy Scientific Tables (5) it is known that men excrete on average ≈16 mmol of creatinine daily (this value being determined from sampling 8 men in the age range of 20–45 years). Then assuming, for the purposes of this calculation, that for any 1 individual, the rates of creatinine and PCS excretion do not vary throughout the day, the maximal rate of PCS urinary excretion observed in our study would be $0.12 \times 0.016 = 0.0019$

mol/day or 0.00024 mol/3- h period. [This agrees reasonably well but not perfectly with the maximum value for "*p*-cresol" urinary excretion given in the Geigy tables (11), where the extreme range is quoted as 0.59–1.08 mmol/day and where there is a note to the effect that *p*-cresol is excreted mainly as the sulfate and glucuronide].

In the absence of other information, the maximal rate of predose *p*-cresol sulfonation is assumed to be the same as the maximal rate of predose PCS urinary excretion (i.e., 0.0019 mol/day or 0.00024 mol/3-h period) and, clearly, this is potentially significant in relation to the difference in average acetaminophen sulfate excretion between the high and low S/G groups.

Consider now the predose (PCS methyl)/(creatinine methylene) integral ratio cutoff of 0.06 (see Fig. 4). With this integral ratio, the PCS/creatinine mole ratio would be ≈0.04 and the assumed predose rate of *p*-cresol sulfonation would be ≈0.04 × 0.016 = 0.00064 mol/day or 0.00008 mol/3-h period. The potential competitive significance of this relative to the difference in average acetaminophen sulfate excretion between the high and low S/G groups probably depends on a cumulative effect of ongoing *p*-cresol sulfonation on sulfonation capacity. Thus, for instance, we would estimate that, over the 9 h preceding acetaminophen administration, 0.00024 mol of *p*-cresol sulfate would be produced by a subject at the 0.06 integral ratio cutoff.

PLS analysis. After repeated model building attempts, using both segmented and full resolution predose spectral data, the only putative PLS-derived finding, which could have arisen by chance, was a weak negative correlation (correlation coefficient −0.321) between predose creatinine (total area normalized segmented predose data) and the total excretion of acetaminophen-related metabolites over the first postdose collection period, with the latter quantity estimated as described in *Supplementary Methods*.

In view of our subsequent findings, the relative lack of success derived from the PLS-based approach, which is used routinely in metabonomics, may potentially be explained by the nonlinearity of the predose to postdose relationship shown in Fig. 4. Thus, it is only when the predose PCS/creatinine integral ratio is high that the S/G ratio may be predicted and the overall predictive power of a PLS model of the data would be expected to be low. Another potentially confounding factor for the PLS analyses would be the partial correlation between *p*-cresol sulfate and phenylacetylglutamine.

PCA of segmented predose ^{1}H NMR spectral data. As described in *Supplementary Methods*, PCA was carried out on the δ 9.1–6.9 region of the original (2003) predose ^{1}H NMR spectra (all subjects included) after first dividing each spectrum into consecutive 0.04 ppm-wide segments, integrating across each segment and normalizing the integrals to give a constant value for a selected spectral region. In 1 case, PCA was performed after the integrals had been normalized to give a constant value for the δ 4.07–4.05 region, which principally contains the creatinine methylene singlet. PCA was also performed after the integrals had been normalized to give a constant value for the δ 3.07–3.03 region, which principally contains the creatinine methyl singlet. In examining the results of these analyses, the 99 subjects were assigned to 3 color-coded classes according to the magnitude of their individual S/G (0–3 h) values. Thus, the 25 subjects with the greatest S/G (0–3 h) values were assigned to class 1 (blue) and the 25 subjects with the lowest S/G (0–3 h) values were assigned to class 3 (red). The remaining 49 subjects were assigned to class 2 (green). As would be expected, very similar results were produced by the 2 normalization methods. In both cases, the optimal class 1-class 3 separation was found on PC2, which represented 5–6% of the variance in each case and was dominated by the spectral segments relating to phenylacetylglutamine (PAG) with a lesser contribution from *p*-cresol sulfate (PCS). With a Bonferroni correction of 30, the *P* value for the 95% level of confidence becomes 0.0017 and the "ranksum" equivalent to the Mann–Whitney *U* test showed significant class 1–class 3 separation on PC2 with *P* values of 0.0013 and 0.0011 being obtained for the 2 normalization methods (creatinine methyl and creatinine methylene), respectively. Selected scores and loadings plots obtained from the PCA of the δ 3.07–3.03 normalized data are shown in Fig. S1.

Subjects identified for exclusion. As noted elsewhere, the usual approach was for the univariate statistical analyses to be performed first with all subjects included and then with a number of subjects excluded. In total, 21 subjects were identified for exclusion because of actual or suspected noncompliance with the study protocol. The particular reasons for making these exclusions were as follows:

One subject was excluded because of an apparently incomplete 0–3 h sample collection, which was contrary to the study protocol and surmised from the postdose NMR data. Fifteen subjects were excluded for potential recent use of acetaminophen, which was contrary to the study protocol and surmised from UPLC-MS analysis of the predose samples. One of these subjects would also have been excluded because the NMR analysis of his predose sample revealed the presence of ketone bodies, which suggested fasting or adherence to an abnormal diet such as the Atkin's diet. This was also contrary to the study protocol, which indicated that the subjects should eat normally. Five subjects were excluded because of a small delay in changing between the 2 postdose collections (noted during study), which was contrary to the study protocol. One of these subjects would also have been excluded for apparent recent consumption of alcohol, which was contrary to the study protocol, and was detected from the NMR analysis of his predose and 0–3 h urine samples.

Numerical data checks (all subjects included).

1. Predose urine

See Table S2

2. Postdose urine

See Table S3 and Table S4

1. Bales JR, Sadler PJ, Nicholson JK, Timbrell JA (1984) Urinary excretion of acetaminophen and its metabolites as studied by Proton NMR spectroscopy. *Clin Chem* 30:1631–1636.
2. Wang Y, et al. (2006) System level metabolic effects of a *Schistosoma japonicum* infection in the Syrian hamster. *Mol Biochem Parasitol* 146:1–9.
3. Spierto FW, Hannon WH, Gunter EW, Smith SJ (1997) Stability of urine creatinine. *Clin Chim Acta* 264:227–232.
4. Masi CM, Rickett EM, Hawkley LC, Cacioppo JT (2004) Gender and ethnic differences in urinary stress hormones: The population-based Chicago Health, Aging, and Social Relations Study. *J Appl Physiol* 97:941–947.
5. Lentner C (1981) *Geigy Scientific Tables* (Ciba-Geigy Ltd, Basel, Switzerland), 8th Ed, Vol 1, pp 63–64.
6. Cloarec O, et al. (2005) Statistical total correlation spectroscopy: An exploratory approach for latent biomarker identification from metabolic 1H NMR data sets. *Anal Chem* 77:1282–1289.
7. Martinez AW, Recht NS, Hostetter TH, Meyer TW (2005) Removal of *p*-cresol sulfate by hemodialysis. *J Am Soc Nephrol* 16:3430–3436.
8. Weljie AM, Newton J, Mercier P, Carlson E, Slupsky CM (2006) Targeted profiling: Quantitative analysis of ^{1}H NMR metabolomics data. *Anal Chem* 78:4430–4442.
9. Broadhurst DI, Kell DB (2006) Statistical strategies for avoiding false discoveries in metabolomics and related experiments. *Metabolomics* 2:171–196.
10. Baker JD (2004) Internatl Patent Appl WO 2004 038602 (A1).
11. Lentner C (1981) *Geigy Scientific Tables* (Ciba-Geigy Ltd, Basel, Switzerland), 8th Ed, Vol 1, pp 90–91.

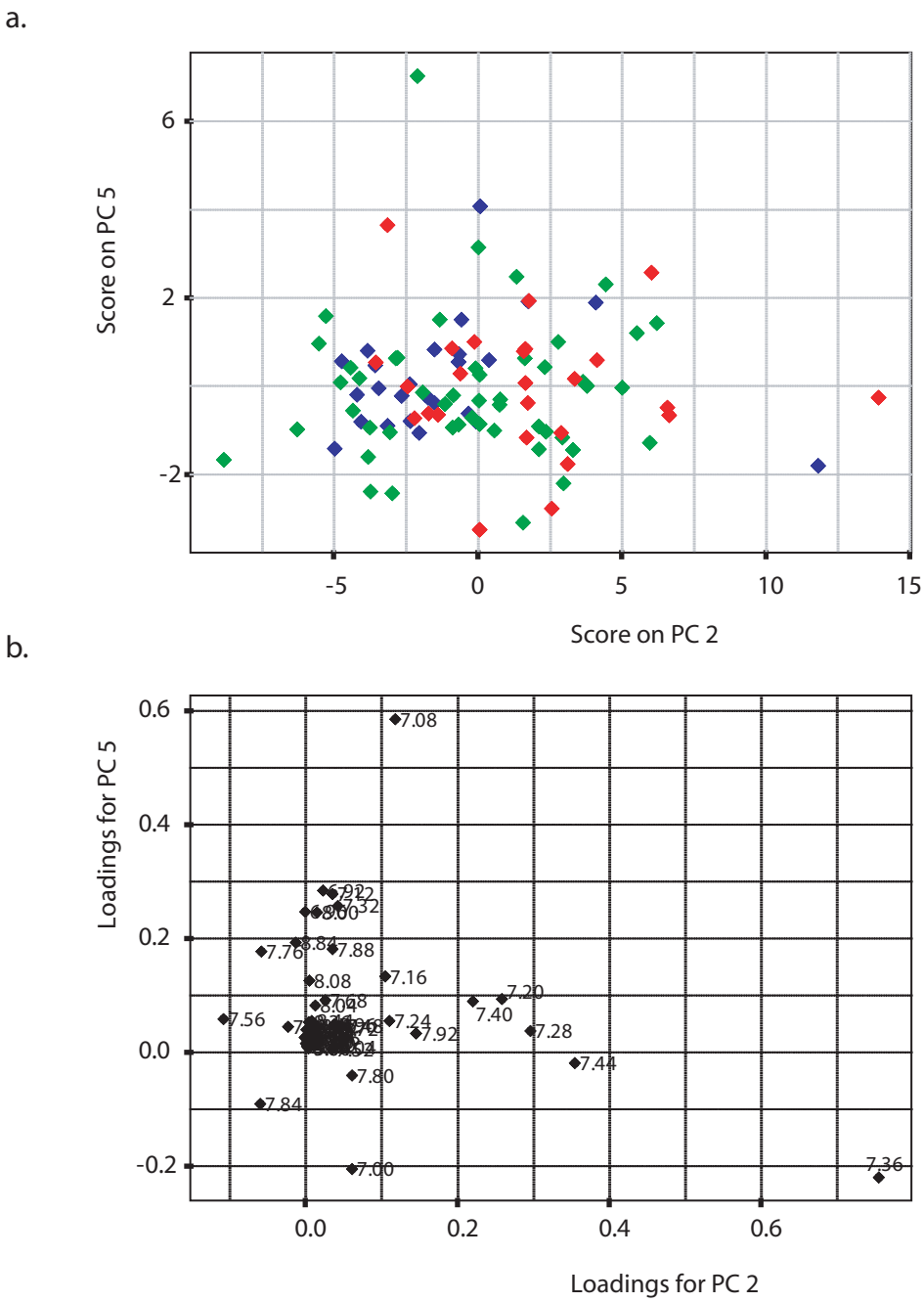

Fig. S1. PCA of the δ 9.1–6.9 region of the predose urinary NMR spectra. (*a*) The scores plot for PC2 vs. PC5 where each point represents a different subject. The points for the 25 subjects showing the highest S/G 0–3 h ratios are colored blue. The points for the 25 subjects showing the lowest S/G 0–3 h ratios are colored red. The points for the 49 subjects showing intermediate S/G 0–3 h ratios are colored green. The plot shows partial separation of the red and blue points on PC2. (*b*) The corresponding loadings plot, with each 0.04 ppm-wide segment identified by the chemical shift at its center. The 7.36, 7.40, and 7.44 spectral segments relate mainly to phenylacetylglutamine (PAG). The 7.20, 7.24, and 7.28 spectral segments relate mainly to *p*-cresol sulfate (PCS). This plot indicates that the spectra producing high scores on PC2 have relatively high levels of PAG and/or PCS. Furthermore, it indicates that the blue points in *a* mainly have relatively low levels of PAG and PCS.

Table S1. Relationships between the measured urinary parameters and age, body mass, and order of sample analysis

	Correlation coefficients for data for all 99 subjects		
	Approx. age, years	Body mass, kg	Order of sample analysis
Predose PCS/creatinine integral ratio	0.122	−0.058	0.028
S/G 0–3 h	0.043	−0.052	0.060
Amount S excreted 0–3 h	−0.017	0.036	0.083
Amount G excreted 0–3 h	−0.039	0.018	0.050
S/G 3–6 h	0.040	−0.047	−0.003
Amount S excreted 3–6 h	−0.042	0.055	−0.002
Amount G excreted 3–6 h	−0.051	0.082	0.002

The predose, 0–3 h, and 3–6 h samples were prepared and analyzed as separate lots. Within each lot, the samples were analyzed in order of increasing subject number. Age was determined approximately as [(year of study) − (year of birth)].

Table S2. Correlation coefficients for the predose urinary data (all subjects included)

	Final PCS/creatinine integral ratios (I.R.) from 2003 NMR spectra	PCS/creatinine ratios from 2007 UPLC-MS and NMR analysis
Initial PCS/creatinine integral ratios (I.R.) from 2003 NMR spectra	0.995	—
Approximate PCS/creatinine integral ratios from MATLAB analysis of 2003 NMR spectra	0.992	—
PCS/creatinine I.R. from 2007 NMR spectra	0.992	0.927

Manual analysis of the 2003 NMR spectra was performed twice (see "initial" and "final" above). The data reported in the paper are those derived from the final (optimized) determination. The MATLAB-derived data are only approximations to the true ratios because local baseline corrections were not applied before performing the integrations. Instead, an overall baseline correction was applied to each spectrum before it was loaded into MATLAB.

In the combined UPLC-MS and NMR analysis performed in 2007, UPLC-MS was used to obtain a measure of the relative amounts of PCS and hippurate in each sample, whereas NMR was used to obtain a measure of the relative amounts of hippurate and creatinine in each sample. The appropriate NMR and UPLC-MS-derived measurements were then multiplied to obtain a measure of the relative amounts of PCS and creatinine in each sample.

Table S3. Correlation coefficients for the 0–3 h urinary data (all subjects included)

	S/G 2003 spectra acetyls (1)	S/G 2003 spectra aromatics	Amt S 2003 spectra acetyls	Amt G 2003 spectra acetyls	7.32 ppm cluster/TSP 2003 spectra	7.15 ppm cluster/TSP 2003 spectra
S/G 2003 spectra acetyls (2)	0.9994					
S/G 2003 spectra aromatics	0.983					
S/G 2007 spectra aromatics	0.982	0.996				
Amt S 2003 spectra aromatics			0.979			
Amt G 2003 spectra aromatics				0.998		
7.32 ppm cluster/TSP 2007 spectra					0.9997	
7.15 ppm cluster/TSP 2007 spectra						0.9995

″(1)″ and ″(2)″ denote the first and second determinations of S/G, respectively, where both determinations were based on the 2003 spectra and integration of the relevant resolution-enhanced acetyls peaks. ″Acetyls″ denotes use of the resolution-enhanced acetyls peaks in the quantitation. ″Aromatics″ denotes that the quantitation was based on the use of the relevant aromatic peaks without resolution enhancement. ″7.32 ppm cluster/TSP″ and ″7.15 ppm cluster/TSP″ denote the relevant integral ratios. The excellent 2003 vs. 2007 correlation coefficients for these parameters indicate reliable sample preparation.

Table S4. Correlation coefficients for the 3–6 h urinary data (all subjects included)

	S/G 2003 spectra acetyls (1)	S/G 2003 spectra aromatics	Amt S 2003 spectra acetyls	Amt G 2003 spectra acetyls	7.32 ppm cluster/TSP 2003 spectra	7.15 ppm cluster/TSP 2003 spectra
S/G 2003 spectra acetyls (2)	0.9993					
S/G 2003 spectra aromatics	0.995					
S/G 2007 spectra aromatics	0.993	0.997				
Amt S 2003 spectra aromatics			0.982			
Amt G 2003 spectra aromatics				0.993		
7.32 ppm cluster/TSP 2007 spectra					0.9995	
7.15 ppm cluster/TSP 2007 spectra						0.9987

″(1)″ and ″(2)″ denote the first and second determinations of S/G, respectively, where both determinations were based on the 2003 spectra and integration of the relevant resolution-enhanced acetyls peaks. ″Acetyls″ denotes use of the resolution-enhanced acetyls peaks in the quantitation. ″Aromatics″ denotes that the quantitation was based on the use of the relevant aromatic peaks without resolution enhancement. ″7.32 ppm cluster/TSP″ and ″7.15 ppm cluster/TSP″ denote the relevant integral ratios. The excellent 2003 vs. 2007 correlation coefficients for these parameters indicate reliable sample preparation.