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*Dedicated to Professor Victor-Emanuel Sahini*
*on the occasion of his 85th anniversary*

# THE EFFECT OF THERAPEUTIC DOSES OF PARACETAMOL AND ASPIRIN ON THE NMR PROFILE OF URINE AT 400 MHZ

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The paper describes the influence of two analgesic drugs on the 400 MHz NMR profile of the urine sample. Although each of the two studied analgesics are known to give rise to several metabolites, due to large discrepancies in the concentrations of these metabolites, only some of them where shown to significantly alter the spectral fingerprint of the urine sample. In the case of paracetamol three metabolites, whereas in the case of aspirin only one metabolite alters the spectral fingerprint if therapeutic doses are used. The spectral regions altered by these metabolites have to be excluded from chemometric data processing if the subjects are known or suspected to use such analgesics as self medication.

## INTRODUCTION

In mid 1980's, Nicholson and Sadler demonstrated the possibility of applying $^{1}$H NMR spectroscopy to biofluids analysis.[1,2]

Since mid 1990's, $^{1}$H NMR spectroscopy became widely used in biomedical research, the technique being accepted as noninvasive, robust and reliable. The current trend in late 1990's and early 21st century is to use chemometric methods for classification of NMR data obtained from biofluids.

Urine analysis by NMR spectroscopy has been used in various applications, including screening or diagnosis of inborn errors of metabolism,[3] monitoring kidney functioning after transplant,[4] urinary tract infection, cancer of organs such as bladder, kidney,[5] or even lung,[6] toxicology studies, diabetes,[7-9] metabolite patterns related to diet,[10] gender and age.[11,12]

Given the complexity of the biofluids, in order to exploit the diagnosis potential of the NMR techniques, it is necessary to identify the spectral "fingerprint" of various compounds which are not disease related, but may be occasionally present in these samples. Among the factors affecting the NMR profile of biofluids one can list foods, drinks, physical effort, age, gender, drugs and the sample treatment prior, during and after NMR analysis. If not well understood, these induced variations can easily lead to erroneous conclusions regarding the medical diagnosis. In order to overcome these variations, different data analysis techniques have been developed,[9,13,14] and there have been a few attempts to propose a standard procedure for NMR analysis of biofluids.[15,16] However, some variations which originate in the natural diversity of different biofluids are still difficult to assess.

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Among the investigated biofluids, urine presents the greatest inter-sample differences regarding metabolite profiles, concentrations and chemical shifts. These variations prevent the automatic assignment of spectral peaks.-One of the factors having an important influence on the NMR profile of the urine samples is the presence of drugs metabolites. Prescription and self administrated drugs, nutrients and other types of pills are used on daily bases by young, adults and elders. As a result, several metabolites of the drugs are excreted in variable concentrations in urine.

In order to characterize the “spectral envelope”, one should first “digitize” the spectrum. The digitization of the spectral envelope for chemometric purposes is usually performed by “downgrading” the spectral resolution, i.e. by treating several data points as a “bulk interval” for either intensity or integral processing. In metabolomics/metabonomics, this process is called “spectral binning” or “bucketing”. If a metabolomics-based technique is to be used for routine diagnostics or as a screening tool, it is imperative to identify the spectral regions which could be affected by drugs and their metabolites.

Different studies have been undertaken involving NMR analyzes of urine for the identification of various metabolites of drugs. In most of the cases, these studies were done on animals, like mice or rats, and the main purpose was to assess the toxicological effect of the drugs.[17] Since NMR spectroscopy has established its place as a qualitative and quantitative tool in metabolomics, studies regarding drug metabolism in humans are currently undertaken by several groups worldwide. Due to the low concentration of the drug metabolites in urine, in some cases it is necessary to use combined techniques like LC-NMR-MS in order to identify these metabolites.

Recently, two papers have addressed the problem of some common self administrated analgesics.[18,19] The papers describe unprescribed use of paracetamol and ibuprofen, by the participants in a large scale human population study.

Acetaminophen or paracetamol is a commonly used analgesic and antipyretic drug. It is used in minor aches and pains, and is the main ingredient in many cold remedies. Due to its extensive usage, it was the first drug whose metabolites were identified in human urine through $^1$H NMR analysis.[20-22]

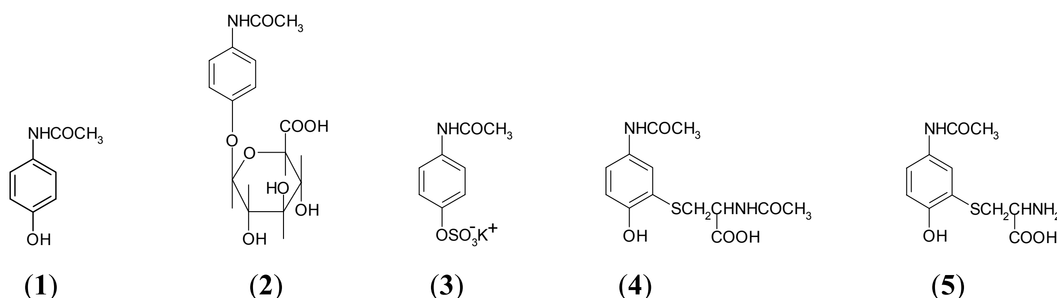
Acetylsalicylic acid or aspirin is another common analgesic and antipyretic drug, used in cases of minor aches, fever or as anti-inflammatory drug. Its metabolites were also identified in the NMR urine spectra in cases of drug overdose.[23-25]

Due to their analgesic and antipyretic properties combined with low toxicity at therapeutic doses, these two drugs are most used as self medication. The NMR profile depends on factors such as magnetic field strength of the spectrometer, pH and ionic matrix, and as there is currently no universally accepted NMR protocol for urine analysis, it is important to evaluate the influence of these drugs on the NMR profile of urine in various conditions. We are describing below the assessment of these influences on the spectral envelopes at 400 MHz, 27°C, under the natural pH of the urine samples. Groups using similar experimental conditions may use these results unfiltered, others should assess again the influence of the metabolites on NMR profile if their protocols differ significantly in terms of magnetic field strength and pH. We have used similar experimental conditions with those described in this paper for our previous studies.[7-9]

## RESULTS

### Paracetamol metabolites

*N*-(4-hydroxyphenyl) ethanamide (paracetamol) (**1**), gives rise to four major metabolites: 4-glucuronosido-acetanilide (**2**), *N*-acetyl-4-aminophenol sulfate (**3**), *N*-acetyl-2-(*N*-acetyl-L-cysteinyl)-4-aminophenol (**4**), and *N*-acetyl-2-(L-cysteinyl)-4-aminophenol (**5**).[26]




Block 1

The typical $^{1}$H NMR spectrum of urine sample is very crowded, with overlapping of most of the peaks corresponding to various metabolites. Due to this reason, combined with the very low excretion concentration, not all the metabolites of paracetamol may be identified in urine by usual $^{1}$H NMR spectroscopy. The two major metabolites, the sulphate (**3**) and the glucuronide conjugate (**2**) as well as *N*-acetyl-2-(L-cysteinyl)-4-amoniphenol (**4**) which is a minor one, are excreted in high enough concentrations, allowing their identification by usual $^{1}$H NMR spectroscopy. Thus, in therapeutic doses only these metabolites contribute to the significant alteration of the NMR profile of urine samples. Other metabolites that may be spotted by special techniques like sample fractionation, freeze drying followed by concentration, hyphenated techniques (e.g. LC-NMR, LC-NMR-MS, SPE-NMR) are not influencing the statistics in which normal $^{1}$H NMR spectra are used. All the assignments for the three metabolites mentioned above were done based on previously reported chemical sifts.[20,21] The $^{1}$H NMR spectra of urine samples from one volunteer before administration of the drug (Fig. 1A) and 7 hours after paracetamol intake (Fig. 1B) are presented below. The major metabolite excreted is the glucuronide conjugate (**2**). For this metabolite several signals can be identified in the $^{1}$H NMR spectrum of the urine sample. The aromatic ring protons give rise to a characteristic *para* substitution pattern, two doublets of triplets centered at 7.36 ppm and 7.14 ppm with coupling constants of 9.0, respectively 2.2 Hz. Another characteristic signal is the doublet at 5.10 ppm (6.6 Hz), corresponding to the *β*-anomeric proton of the glucuronide group. The rest of the protons from the sugar ring give rise to signals between 3.30 and 4.00 ppm, in a very crowded region of the spectrum, making thus impossible the assignment of the other individual signals. The *N*-acetyl protons give a singlet signal at 2.16 ppm.

The second major metabolite, the sulfate conjugate (**3**), has three characteristic signals. A *para* substitution pattern formed from two doublets of triplets centered at 7.45 and 7.32 ppm, with coupling constants of 9.0 and 2.1 Hz, can be assigned to the aromatic ring protons. The *N*-acetyl protons give a singlet signal at 2.18 ppm.

*N*-acetyl-2-(L-cysteinyl)-4-amoniphenol (**4**), the third metabolite of paracetamol, could be identified through the singlet at 1.86 ppm. This signal corresponds to the side-chain *N*-acetyl group.

## Aspirin metabolites

The main metabolites of 2-acetoxybenzoic acid (aspirin) (**6**) are: 2-hydroxybenzoic acid (salicylic acid) (**7**), 2-(2-hydroxybenzoyl)-aminoacetic acid (salicyluric acid) (**8**), 2,5-dihydroxybenzoic acid (gentisic acid) (**9**), *ortho* carboxyphenyl-glucuronide (**10**), and *ortho* hydroxybenzyl-glucuronide (**11**).[27, 28]

Aspirin (**6**) is rapidly hydrolyzed in vivo to salicylic acid (**7**), which in turn is converted mainly to salicyluric acid (**8**). The other metabolites (**9-11**) account for less than 15% metabolic pathways of the drug.

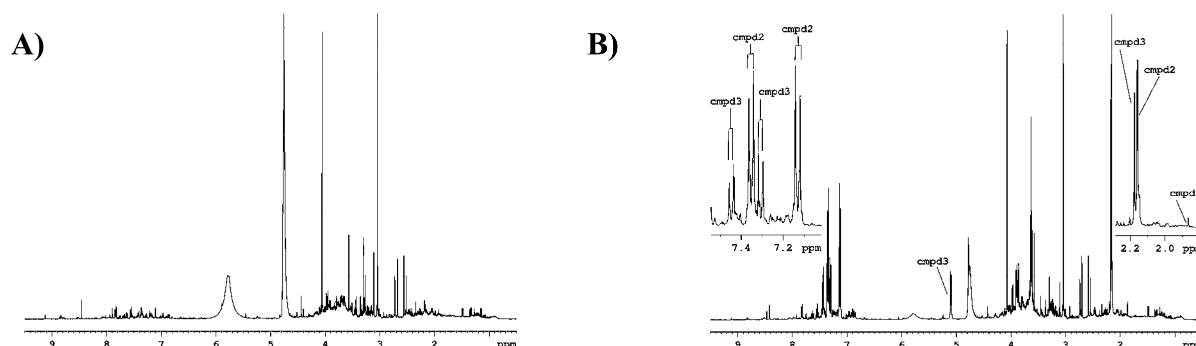
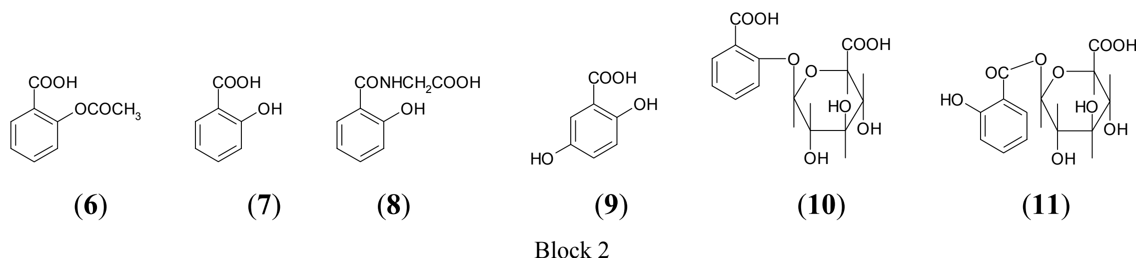



Fig. 1 – $^{1}$H NMR spectra of **A)** urine sample before paracetamol administration and **B)** 7 hours after paracetamol administration. The signals corresponding to the major three metabolites are assigned. *Cmpd2* refers to 4-glucuronosido-acetanilide (**2**), *cmpd3* to *N*-acetyl-4-aminophenol sulfate (**3**), and *cmpd4* to *N*-acetyl-2-(*N*-acetyl-L-cysteinyl)-4-aminophenol (**4**).




Block 2

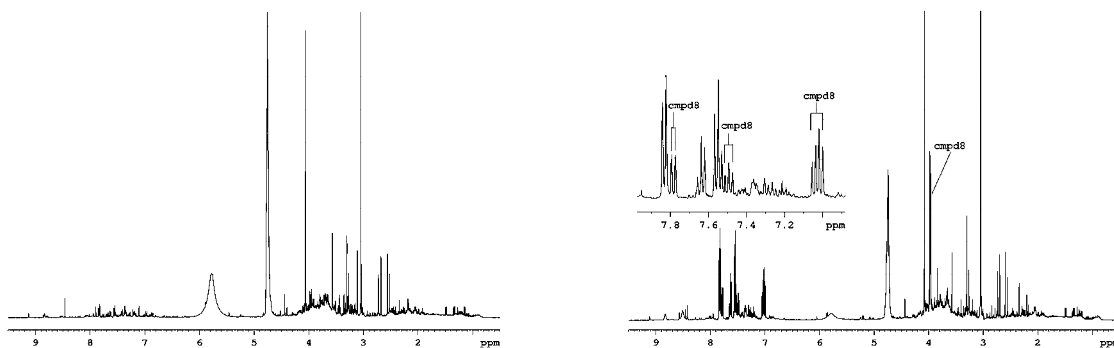



Fig. 2 – ^{1}H NMR spectra of **A)** urine sample before aspirin administration and **B)** 7 hours after aspirin administration. The signals corresponding to salicyluric acid (**8**) are assigned.

The ^{1}H NMR spectrum of urine sample from one volunteer before and after aspirin intake is presented in Fig. 2. In this case, only the signals corresponding to the major metabolite, respectively salicyluric acid (**8**), could be identified. Similarly with the discussion related to paracetamol (above), the identification of only one metabolite of aspirin in the normal ^{1}H NMR spectrum of urine is due to the low concentration of the rest of metabolites when therapeutic dose is administrated. Other metabolites, present in the sample do not significantly affect the NMR envelope and are of no concern for chemometric studies.

Salicyluric acid (**8**) presents 4 distinctive groups of signals. The protons from the methylene group give rise to a doublet in the 3.95-4.00 ppm interval, but it is overlapping with the signal from hippuric acid, a metabolite which is normally present in urine. The aromatic protons have the following characteristic signals: a doublet of doublets, centered at 7.78 ppm (7.9 and 1.5 Hz), a triplet of doublets, centered at 7.50 ppm (8.5 and 1.7 Hz) and a group of overlapping signals between 6.99 – 7.06 ppm corresponding to the other two aromatic protons.

## DISCUSSION

The "fingerprint" of the studied drugs in the ^{1}H NMR spectra of urine samples is visible for a long period of time, and the shape of the "fingerprint" is variable in time. Thus, several signals of the paracetamol metabolites were still present after 24 hours from the drug intake, but at 38 hours only the signals corresponding to the *N*-acetyl protons were visible. In the first hours, the glucuronide derivative (**2**) has the most intense signals, but after 15 hours the ratio glucuronide (**2**):sulfate (**3**) becomes closer to 1. The *N*-acetyl-2-(L-cysteinyl)-4-amoniphenol (**4**) is excreted in much lower concentration. Due to this reason, its signals, especially the singlet at 1.86 ppm, could be identified in the ^{1}H NMR spectra only up to 20 hours from the drug intake. For both volunteers, based on the NMR data, the urinary excretion of paracetamol major metabolites was complete within approx. 38 hours.

The metabolites quantification was done using the signals corresponding to the protons from the *N*-acetyl groups. The aromatic signals are overlapping with other signals, thus the quantification error is larger for these signals and they were not used for concentration determinations. Signals intensities were used to calculate the concentrations and the obtained results were displayed in the concentration *vs*. time plot, presented in Fig. 3A. To calculate these concentrations, we used the TSP signal intensity, its final concentration in the urine samples being 0.5 mM.

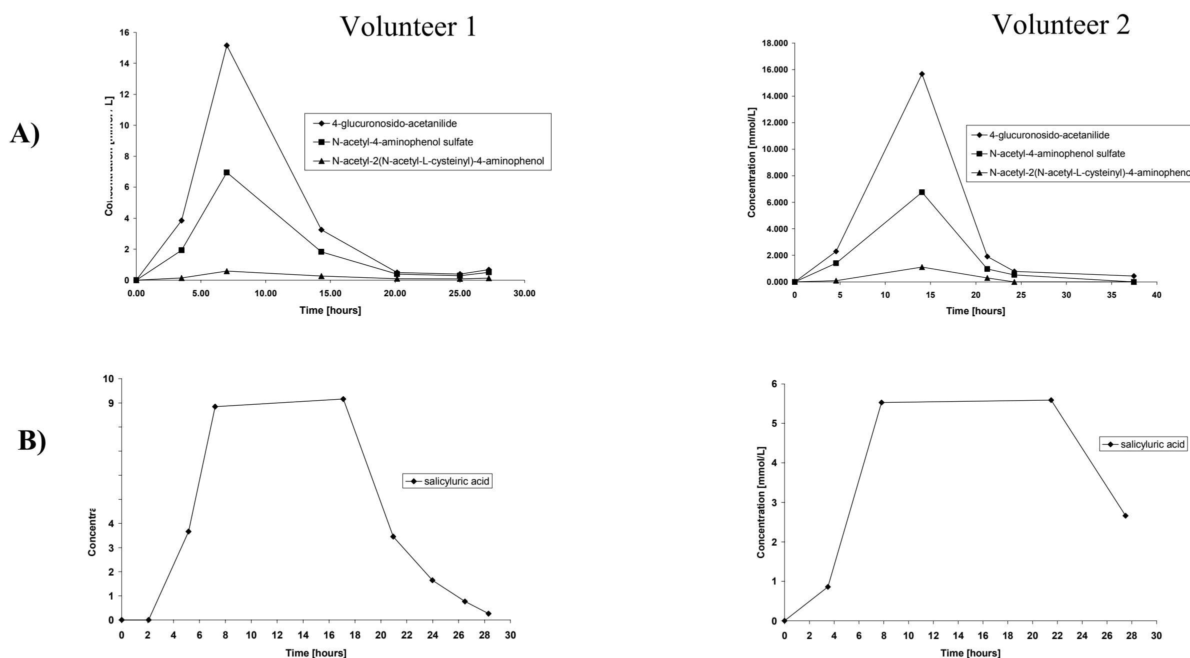



Fig. 3 – Evolution of the metabolites concentrations as obtained from the $^1$H NMR data after administration of **A)** paracetamol and **B)** aspirin.

In the case of aspirin intake, in the $^1$H NMR spectra of urine samples, the only identifiable signals in the normal $^1$H NMR spectrum were those of salicyluric acid (**8**). The other metabolites are excreted in a much lower concentration, and do not affect the profile of the NMR envelope. From the $^1$H NMR spectra, the urinary excretion of the aspirin metabolite was completed within 28 hours. Similarly with the previous case, the salicyluric acid concentration was calculated based on signals intensities. For these calculations only the signal from 7.78 ppm was considered. The obtained results were displayed in the concentration *vs*. time plot, presented in Fig. 3B. The plot shows a rapid increase of concentration for the excreted salicyluric acid within the first 7-8 hours, with a maximum in the first 20 hours, followed by a slower decrease to total disappearance, after 28 hours from the drug intake.

## EXPERIMENTAL

### Sample collection

Samples have been collected from two healthy women, aged 22 and 29, without diet restriction. Urine samples were collected from each volunteer prior the administration of therapeutic doses of paracetamol and aspirin. All urine samples post dose were collected over 40 hours after the drug administration. Aliquots of 2 mL were stored in a freezer at -20°C until NMR analysis, but not more than one week. The two drugs were administrated at two months time interval.

### Sample preparation

Prior to NMR analysis, the urine samples were allowed to thaw at room temperature for 30 minutes. A volume of 1 mL urine was centrifuged for 10 minutes at 7000 rotations per minute in order to remove any solid particles. For the NMR analysis 540 μL of the supernatant was mixed with 60 μL of 5 mM sodium 3-(trimethylsilyl)-[2,2,3,3-d4]-1-propionate (TSP) in $D_2O$ solution. All 600 μL of sample were transferred into a 5 mm NMR tube and subjected to the analysis.

### NMR measurements

$^1$H NMR spectra were acquired at 300.15 K, on a Bruker Avance III 400 MHz spectrometer, operating at 400.13 MHz, using a 5 mm inverse detection probe equipped with gradients on the *z*-axis. The samples were run in 5 mm Wilmad 507 NMR tubes. The spectra have been recorded with the noesy presaturation pulse sequence using 32 scans, 30 s relaxation delay, 4 s acquisition time, 8223 Hz spectral window, collecting 64 K data points, with a resolution of 0.12 Hz. An exponential line broadening factor of 0.3 Hz was used in post-acquisition FID processing. The chemical shifts are reported as δ values (ppm) referred to TSP (0.0 ppm) as internal standard.

## CONCLUSIONS

The $^1$H NMR profile of urine samples is influenced by the paracetamol and aspirin metabolites. The signals corresponding to these metabolites can be observed in the $^1$H NMR spectra up to 38 hours in the case of paracetamol, and up to 28 hours for aspirin, from the moment of the drug's therapeutic dose administration.

When applying "spectral binning" to the NMR data, in case of knowledge of paracetamol or aspirin intake, the following regions should be excluded: 1.85-1.87, 2.15-2.17, 3.30-4.00, 5.09-5.10, 7.12-7.16, 7.30-7.37, 7.43-7.46 ppm for paracetamol intake, and 3.96-3.98, 7.00-7.06, 7.47-7.51, 7.77-7.79 ppm for aspirin intake.

Due to the fact that in the case of paracetamol several metabolites contribute to the drug's fingerprint, while in the case of aspirin only one metabolite contributes to the fingerprint, the time behavior of the spectral envelope is different in the two studied cases. In the first case, the shape of the fingerprint is variable in time, until it eventually vanishes, while in the second case the shape of the fingerprint is constant in time, the only change being its intensity variation, with initial increase and final gradual disappearance.

If one tries to develop algorithms for automatic identification by NMR of drugs signatures in urine, e.g. by pattern recognition techniques, apart from other variables (like pH), in some cases the variation of the shape of the fingerprint in time should be also taken into account.

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