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Characterization of a paracetamol metabolite using on-line LC-SPE-NMR-MS and a cryogenic NMR probe

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Abstract

In this study, the hyphenation of LC-SPE-NMR-MS at 500 MHz was applied to the structural elucidation of a low concentrated paracetamol metabolite present in human urine. Single or multiple peak trapping of the mass detected metabolite on SPE cartridges was employed to increase the sensitivity and quality NMR measurement over the conventional LC-NMR method. After the elution of the metabolite from the SPE cartridge to the NMR flow probe using deuterated acetonitrile for initial NMR investigation, the fraction was revovered by flushing the sample out of the NMR probe head with nitrogen gas. On the recovered fraction, high resolution FT-ICR-MS measurements were conducted, giving exact mass information about the unknown metabolite. In addition, a cryogenic NMR micro probe head was used to enhance the sensitivity of the NMR measurement by a factor of 5 in order to run 2D experiments for structural elucidation of the unknown metabolite. The combination of both MS and NMR results, led unequivocally to the elucidation of the structure as the ether glucuronide of 3-methoxy-paracetamol.
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1. Introduction

High field NMR spectroscopy is a very powerful tool and efficient method for the direct investigation of xenobiotic metabolites in complex matrixes, i.e., urine, plasma and bile [1–5]. The unambiguous identification of drug metabolites can usually only be accomplished by spectroscopic characterization, generally mass spectroscopy (MS) and nuclear magnetic resonance (NMR) spectroscopy. By use of directly coupled stopped flow LC-NMR at 800 MHz even components at very low concentration levels can be identified. The first study presenting the use of directly coupled 800 MHz for the analysis of drug metabolites was published by Sidelmann et al. [6].

Solid phase extraction (SPE) has been proven to be very successful for the selective removal of endogenous compounds in biofluids and thereby aid the structure elucidation of unknown drug metabolites [7,8]. SPE is normally used in front of a separation to preclean or to preconcentrate samples (SPE-LC) prior to injection. Several studies report the use of SPE off-line, prior to chromatographic analysis, e.g., for the removal of endogeneous compounds in urine to facilitate studies of drug metabolites [6,7]. In these cases, SPE is used to concentrate the sample before the separation on a HPLC column, which is then connected to the NMR probe. Another combination of SPE, HPLC and NMR was used to increase the sensitivity of HPLC-NMR analysis by concentrating the eluted compounds on a guard column [9,10].

Examples of off-line SPE followed by on-line LC-NMR coupling include the analysis of natural products [11–13]. One of the latest technological improvements was the recently introduced LC-SPE-NMR setup [14–16]. In the case described here, SPE is used on-line after the analytical column to trap LC peaks. One of the latest developments in the field of hyphenated techniques, the utilization of an

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automated SPE system as interface between liquid chromatography (LC) and NMR, is demonstrated.

The identification of metabolites of paracetamol in human urine by directly coupled LC-NMR was one of the first examples to show the efficiency of this technique. Major metabolites are known and have already been described in the literature [5,17,18]. The objective of this study is to apply newly developed techniques such as LC-NMR-MS or LC-SPE-NMR to the investigation of paracetamol metabolites. This study includes the use of Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) for exact mass determination of unknown constituents and LC-SPE-NMR to increase the sensitivity of the measurement at lower fields (e.g., 400 or 500 MHz) and to eliminate HPLC solvent signals.

2. Experimental

2.1. Chemicals

All compounds used in the experiments were purchased from Sigma–Aldrich (Deisenhofen, Germany), except for the HPLC solvents. Acetonitrile was purchased from Riedel-de Haen (Seelze, Germany) and high purity HPLC-grade water was obtained from Carl Roth GmbH (Karlsruhe, Germany). The deuterated solvents were purchased from Deutero GmbH (Kastellaun, Germany).

2.2. Dosing and urine collection

Urine was obtained from a normal healthy male 4 h after an oral dose of 1 g of paracetamol. Samples were stored frozen at −38 °C until analysis. The urine was injected directly onto the LC column without any further pretreatment of the sample.

2.3. LC-SPE-NMR-MS at 500 MHz

2.3.1. Chromatography

A 1100 series chromatographic system including a quaternary pump, column thermostat, autosampler and variable wavelength detector from Agilent (Waldbronn, Germany) was connected to an NMR/MS interface from Bruker (Bruker BioSpin, Rheinstetten, Germany). Five percent of the eluent was split to an Esquire 3000 iontrap multipole mass spectrometer equipped with an electrospray interface from Bruker Daltonik (Bremen, Germany) and operated in positive ionisation mode. The major fraction of the eluent (95%) followed the flow path to a Prospekt 2 automated solid phase extraction unit from Spark Holland (Emmen, Holland). Chromatography was performed using a YMC-Pack J'Sphere H80 column (250 mm × 2.0 mm i.d, 4 μm particle size) from YMC Europe (Schermbeck, Germany). Gradient elution was performed using eluent A (H_2O + 0.1% formic acid) and eluent B (acetonitrile) with the following linear gradient: 1% B held over 5 min to 30% B within 60 min and to 100% B within 75 min. The flow rate used was 0.2 mL/min and the separation was carried out at 10 °C to improve the separation of the unknown metabolite from closely eluting peaks.

2.4. SPE-procedure

The peaks of interest were detected using the UV response at 254 nm and the mass response at *m*/*z* 358. Solvents used were non-deuterated, saving cost and preventing the exchange of acidic protons like –OH, –NH, $–NH_2$ with D_2O and, therefore simplify mass spectrometric analysis. A make-up flow of eluent A was added to the post column eluate in a ratio of 4:1 in order to improve the retention on the Hy sphere GP cartridge (10 mm × 2 mm) from Spark Holland (Emmen, Holland).

After the trapping process, the cartridges were dried with nitrogen to remove residual solvents. Pure deuterated acetonitrile was used to flush the peak from the SPE cartridge directly into the NMR cell. The S/N gain for the SPE-NMR over conventional LC-NMR depends on the probe volume, the peak broadening during transfer and the ratio of the volume of the original LC peak to the volume of the elution band.

2.5. NMR spectroscopy

From the SPE cartridges the collected fractions were eluted into a Bruker AVANCE 500 MHz NMR spectrometer operated at 500.13 MHz, equipped with a 3.0 mm $^1H/^{13}C$ inverse LC flow probe (active volume 60 μL).

Routine measurements were carried out using multiple solvent suppression [19,20] with time-shared double presaturation of the water and the acetonitrile ^1H frequencies by means of a shaped low power rf-pulse and CW-decoupling on the F2-channel for the decoupling of the ^{13}C satellites of acetonitrile. The data were collected into 32 K computer data points with a spectral width of 10,000 Hz, 90° pulses were used with an acquisition time of 1.6 s, and the spectra were acquired by accumulation of 128 scans for the triple trapping (1024 scans for the single trapping). Prior to Fourier transformation, an exponential multiplication was applied to the FID, corresponding to a line broadening of 1 Hz.

2.6. FT-ICR-MS

All experiments were performed using an APEX II Fourier transform ion cyclotron resonance mass spectrometer (Bruker Daltonics Inc., Billerica, MA, USA) equipped with a 7 T superconducting magnet (Bruker Biospin GmbH, Rheinstetten, Germany). A cylindrical "infinity cell" [21] type ICR trap was used with equipotential-line-segmented trapping plates. The electrospray mass spectra were acquired using an APOLLO electrospray ion source (Bruker Daltonics Inc., Billerica, MA, USA) in negative ion mode in the mass range from *m*/*z* 55 to 1500 using 512 K data points. External calibration of the spectra was accomplished by measuring

a homologous series of *p*-hydroxy benzoic acid esters. Acquisition and post-processing of MS data have been carried out using the software XMASS 5.0 (Bruker Daltonics, Billerica, MA, USA). For the infusion experiments, the recovered peaks from the SPE-NMR run were dissolved in acetonitrile and 0.1% formic acid (50:50, v/v). Samples were introduced by direct infusion using a syringe pump (Cole Parmer, Vernon Hills, IL, USA) at a flow rate of 60 μL/h.

2.7. Off line measurement from recovered SPE fraction in a 3 mm cryogenic probehead at 600 MHz

The set-up consisted of a Bruker AVANCE 600 MHz spectrometer equipped with a 3.0 mm dual inverse Cryoprobe™ (active volume 59 μL). The samples were recovered from the previously described LC-SPE run. The sample was obtained by repeating the chromatography and trapping the unknown paracetamol metabolite peak three times. After drying the SPE cartridge with nitrogen, the sample was eluted directly into a 3 mm sample tube with 150 μL deuterated acetonitrile.

2.8. NMR measurements

A phase-sensitive 2D NOESY experiment [22–24] was carried out with the unknown paracetamol metabolite on a Bruker AVANCE 600 MHz spectrometer equipped with a 3.0 mm Cryoprobe™ (active volume 50 μL). ^{1}H NMR spectra were obtained at 600.23 MHz. Chemical shifts were referenced to that of acetonitrile defined as 2.0 ppm. A total of 181 increments with 128 transients and 2 K computer data points were acquired in simultaneous mode with a spectral width in both dimensions of 6127.5 Hz. With an acquisition time of 167 ms and an applied mixing time of 2 s, the total acquisition time was 20 h. The data were apodized with a shifted squared sine bell window function (2.5 Hz) in both dimensions and zero-filled in the Fi dimension to 1024 data points.

3. Results and discussion

Chromatographic separation of the individual metabolites was readily achieved by HPLC using gradient methods. Fig. 1 presents the separation of human urine (50 μL injection) after dosage of 1 g paracetamol. Small amounts of unchanged paracetamol (peak 3) can still be observed. Interpretation of the MS/MS data obtained during optimisation of the chromatography revealed the presence of an unknown metabolite in the sample showing a mass difference of 30 in comparison with the known glucuronide of paracetamol. However, the concentration was too low for structural elucidation at 500 MHz.

To gain the exact mass of the unknown metabolite, high resolution FT-ICR-MS [25,26] measurements were carried out on peaks recovered from the SPE experiments.

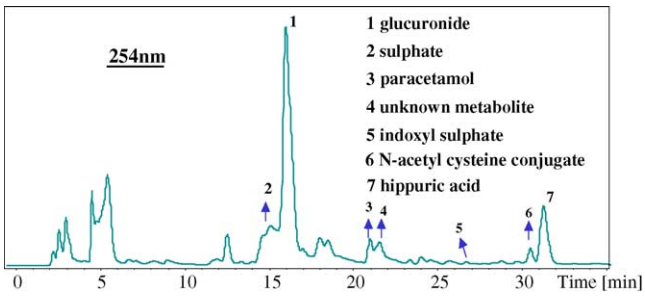

Fig. 1. HPLC chromatogram of paracetamol metabolite in human urine. Peak 1: glucuronide; 2: sulphate; 3: paracetamol; 4: unknown paracetamol metabolite; 5: indoxyl sulphate; 6: *N*-acetyl cysteine conjugate; 7: hippuric acid.

The fractions which correspond to peaks 1 and 4 in Fig. 1 were infused into the FTMS and the mass spectra are shown in Fig. 2c and d, respectively, while Fig. 2a and b shows the result from first LC-NMR/MS runs performed with D_2O instead of water using the Esquire 3000. The nominal masses obtained from this experiment can be used for the calculation of the number of exchangeable protons as visualized in Fig. 2a which indicates that both, the known and unknown paracetamol glucuronides own five exchangeable protons. The FT-MS measurements were carried out in non-deuterated solvents, therefore, exact masses could be obtained. For the known paracetamol glucuronide (peak 1) an, m/z = 326.0881 $[M - H]^-$ was expected (found at 326.0880; mass error: 0.3 ppm). For the unknown metabolite a mass of m/z = 356.0991 was found, which is in good agreement with the proposed molecular formula of $C_{15}H_{19}NO_9$ of the ether glucuronide of 3-methoxy-paracetamol (expected: 356.0987 $[M - H]^-$; mass error: 1.0 ppm).

The formation pathway for this metabolite is postulated in Fig. 3. Paracetamol is first hydroxlated at position 3. The enzyme catechol O-methyl transferase (COMT) then catalyses the transfer of a methyl group from the coenzyme S-adenosylmethionine to the 3-position hydroxyl group [27]. The phenol group at position 4 then undergoes conjugation to give the ether glucuronide of 3-methoxy-paracetamol.

At the lower NMR field strength of 500 MHz, it was possible to obtain a ID proton NMR spectrum of the new metabolite with 1024 scans after a single trapping step with an injection volume of only 50 μL. The use of the newly developed LC-SPE-NMR interface, with its possibility to multiply trap the same peak from several injections on one cartridge, can help to overcome sensitivity problems. After trapping the same peak out of three injections on the same cartridge it was possible to reduce the required number of scans from 1024 to 128 to get an interpretable NMR spectrum as shown in Fig. 4b. A major advantage of this approach is the fact that the drying process removes most of the water from the cartridge leading to the detection of the exchangeable amide proton of the ether glucuronide of 3-methoxy-paracetamol. This proves the new glucuronide to be an oxygen rather than a nitrogen bound glucuronide.

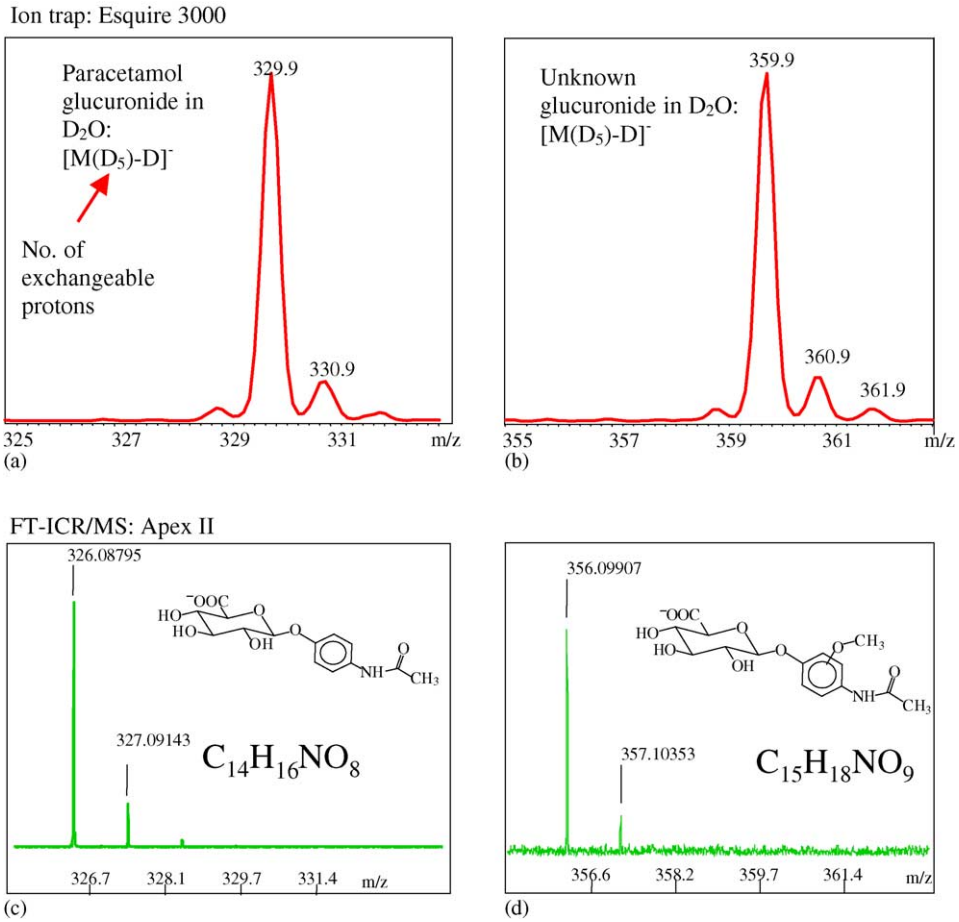

Fig. 2. Esquire 3000 LC-NMR/MS result with D_2O instead of water allowing the calculation of the number of exchangeable protons (a) paracetamol glucuronide and (b) unknown metabolite, and Apex II FT-ICR/MS determination of exact mass measurements using CH_3CN + 0.1% formic acid with 60 μL/h infusion. (c) Paracetamol glucuronide and (d) unknown paracetamol metabolite.

Further, gain in sensitivity was achieved using a cryoprobe. The recovery of the measured fraction into an NMR tube was easily achieved by backflushing the LC probe head with nitrogen gas. This reduced the danger of further dilution or contamination with proton carrying solvents. The fraction recovered in a 3 mm NMR tube was transferred to a 600 MHz spectrometer equipped with a cryogenic probe head. The gain of NMR sensitivity now enabled the acquisition of a full 2D NOESY experiment within 20 h. The proton spectrum on top of the NOESY spectrum in Fig. 5 shows a 1,3,4-trisubstituted pattern in the aromatic region with a doublet at 7.4 ppm showing a meta coupling, a doublet at 7.04 ppm indicating a large otho coupling and a doublet of doublet at 6.98 ppm with an ortho and meta coupling besides two signal groups resulting from the AA′ BB′ spin system of un-metabolised paracetamol. In the sugar region, a dou-

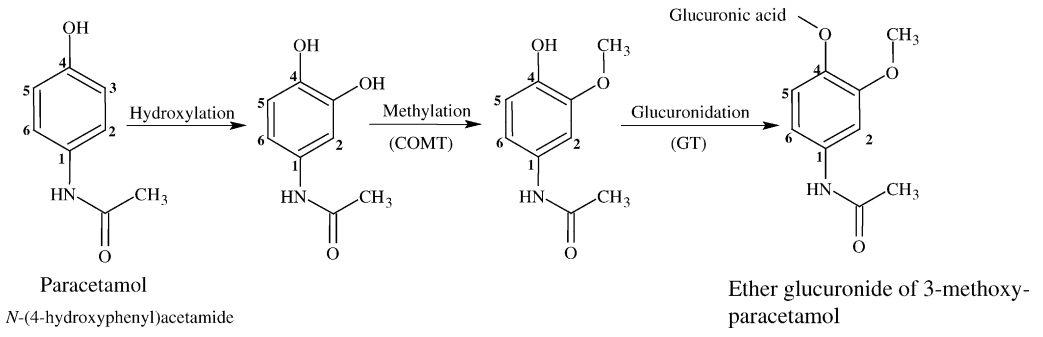

Fig. 3. One probable mechanism that leads to the ether glucuronide of 3-methoxy-paracetamol.

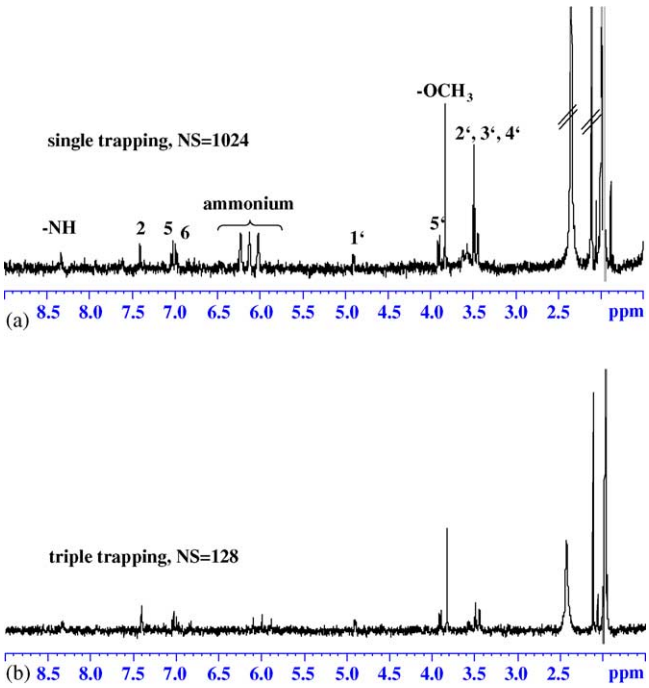

Fig. 4. Fifty microliters paracetamol in human urine was injected for SPE trapping. (a) Shows the result after single trapping and (b) the result after tripple trapping.

blet at 4.8 ppm with a large coupling constant corresponds to the anomeric proton of the glucuronic acid moiety. The singlet at 3.8 ppm shows a chemical shift typical for methoxy groups.

A correlation in the NOESY spectrum in Fig. 5 between the methoxy group at 3.8 ppm and the aromatic proton at 7.4 ppm proves the close spatial relationship between both protons.

A second strong correlation can be observed between the anomeric proton resonating at 4.8 ppm and the doublet showing the large ortho coupling constant at 7.04 ppm as indicated in Fig. 5.

It has to be emphasized that the anomeric proton shows only one correlation to a proton in the aromatic region. This strongly points to the proposed structure with a methoxy group being in the three-position rather than a second possible structure where the methoxy group would be in the two-position, which would also give a NOESY correlation between the methoxy group and a doublet with the small meta coupling in the aromatic region.

However, this molecule would not show a correlation between the anomeric proton and the doublet with a meta coupling only.

Instead, an ether glucuronide of 2-methoxy-paracetamol would show a strong NOESY correlation between both the methoxy group and the anomeric proton to a doublet in the aromatic region with a small meta coupling (H3). In addition one would expect a correlation between the anomeric proton and the second adjacent aromatic proton in this molecule

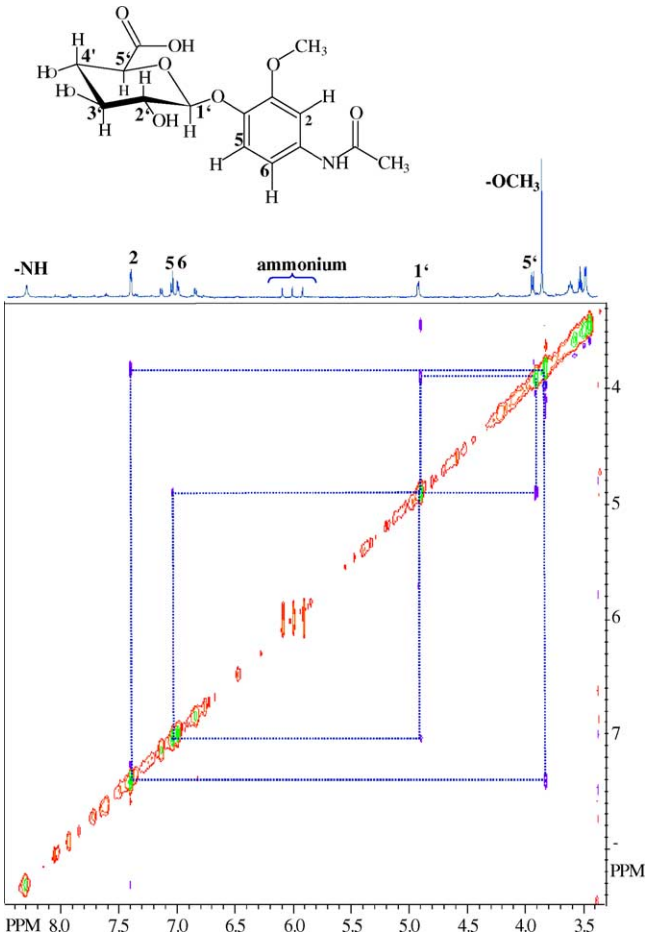

Fig. 5. 2D NOESY spectrum (mixing time = 2 s) of the ether glucuronide of 3-methoxy-paracetamol at 600 MHz in a 3 mm cryogenic probe head (total experiment time 20 h). The sample was recovered from a conventional 3 mm LC probe head after a triple trapping SPE-NMR run (result shown in Fig. 4b).

showing a doublet of doublet with an ortho and meta coupling (H5).

4. Conclusion

The LC-SPE-NMR coupling allows to increase the sensitivity of a hyphenated system over conventional LC-NMR by a factor of at least 2. Quantitative enrichment on the SPE cartridges enables multiple trapping steps increasing the NMR sensitivity by additional factors. As the chromatography is performed with standard HPLC solvents, the exact mass information can be deduced. The drying and elution step with pure deuterated acetonitrile allows the detection of most exchangeable protons in the NMR spectrum if no presaturation of the residual water resonance is applied. In addition, the sample can be recovered without further dilution or contamination by flushing the LC-probe head with nitrogen once the routine NMR measurement is finished. Recovering of the peak into an NMR tube enables the re-investigation of the fraction in a cryogenic probe increasing the sensitivity of the

NMR measurement by a factor of 5–6. A small portion of this fraction can be diluted with aqueous buffer to perform accurate MS measurements yielding the sum formula of the analyte.

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