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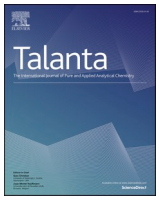

Application of deep UV resonance Raman spectroscopy to column liquid chromatography: Development of a low-flow method for the identification of active pharmaceutical ingredients

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ABSTRACT

In this study, deep UV resonance Raman spectroscopy (DUV-RRS) was coupled with high performance liquid chromatography (HPLC) to be applied in the field of pharmaceutical analysis. Naproxen, Metformin and Epirubicin were employed as active pharmaceutical ingredients (APIs) covering different areas of the pharmacological spectrum. Raman signals were successfully generated and attributed to the test substances, even in the presence of the dominant solvent bands of the mobile phase. To increase sensitivity, a low-flow method was developed to extend the exposure time of the sample. This approach enabled the use of a deep UV pulse laser with a low average power of 0.5 mW. Compared to previous studies, where energy-intensive argon ion lasers were commonly used, we were able to achieve similar detection limits with our setup. Using affordable lasers with low operating costs may facilitate the transfer of the results of this study into practical applications.

1. Introduction

Precise and economic quality control (QC) methods are of paramount importance in the pharmaceutical industry [1,2]. They ensure drug safety and efficacy, regulatory compliance, and address the challenges posed by a rapidly evolving global landscape. As supply chains become more globalized, maintaining product quality across diverse locations becomes a daunting task for companies [3,4]. Competitive pressure underscores the need to implement efficient QC methods to reduce costs and increase competitiveness. Furthermore, the pharmaceutical industry has shifted its focus toward the development of green analytical methods due to increasing economic, social and environmental concerns [5,6]. Within these continuously altering boundaries, technological breakthroughs offer new opportunities for more precise and effective analytical methods that can take pharmaceutical quality assurance to an unprecedented level. The development and improvement of new methods for HPLC that allow the identification of APIs can make a decisive contribution to this.

Currently, HPLC combined with UV/Vis spectroscopy is commonly used for drug content analysis [7,8]. However, this method is not able to identify compounds by retention time and UV/Vis spectral information unambiguously. Hence, there is a substantial demand for detectors capable of performing compound identification. Mass spectrometry, often regarded as a tool for structural elucidation, is not a standard choice for this purpose due to factors such as high costs and specialized expertise requirements [8,9]. In this context, alternative approaches like Raman spectroscopy come into consideration [10,11].

Raman spectroscopy provides a promising solution, particularly for structural identification using spectral databases [12,13]. However, Raman spectroscopy is a comparatively insensitive method [14]. To overcome this limitation, there is a fundamental interest in the development of sensitive Raman detectors capable of identifying substances even at low concentrations. Several methods have been developed to increase sensitivity, such as confocal Raman microscopy (CRM), resonance Raman spectroscopy (RRS), surface-enhanced Raman spectroscopy (SERS), or the application of liquid core waveguides (LCW) [15,16]. UV resonance Raman spectroscopy (UV-RRS) is a specific form of RRS [16]. UV-RRS provides the potential to revolutionize structural identification in pharmaceutical quality assurance and offers a more efficient analytical approach.

Previous studies have shown that UV-RRS provides higher sensitivity and fluorescence-free Raman spectra at excitation wavelengths below 250 nm, also known as deep UV resonance Raman spectroscopy (DUV-RRS) [17]. For this low wavelength, the laser market for Raman

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applications is still very limited. As an example, Dijkstra et al. employed a frequency-doubled argon-ion laser for the coupling of DUV-RRS to an HPLC [18]. While the analyte molecules are excited resonantly, such an effect is not observed for common HPLC solvents due to the absence of chromophores. This unilateral resonant excitation makes DUV-RRS particularly interesting for the coupling with HPLC, since in non-resonant Raman spectroscopy the signals of low-concentrated analytes are often superimposed by the strong Raman signals of the solvents.

However, the high acquisition and operating costs of frequency-doubled argon-ion lasers make such systems unattractive for many laboratories [19]. Argon-ion lasers operate at an electrical power consumption of up to 12.5 kW, delivering a laser output of about 60 mW at 248.2 nm (Laser system: Innova 90C FreD, Coherent Inc., US). The high power consumption and the need for a cooling system make this laser ecologically and economically questionable and resource-intensive [20].

This study investigated an alternative laser technology with the aim of finding a more environmentally friendly and cost-effective excitation source for deep UV resonance Raman applications. Therefore, a metal vapor hollow cathode soft-pulse laser with an excitation wavelength of 248.6 nm and a calculated continuous wave (CW) power of 0.5 mW was applied. In comparison to the previously mentioned argon-ion laser, the energy consumption of the hollow cathode laser is less than 10 W. This implies that the argon-ion laser exhibits 1250 times higher energy consumption. In terms of energy efficiency, the hollow cathode laser performs a ten times higher conversion rate from electrical power to emitted laser power. However, the comparison of the lasers only serves as a rough estimate, as different laser technologies and emission wavelengths were used.

To counteract the comparatively low laser power, a low-flow method was developed in this study to increase the measurement time. This is a common approach, similar to the stop-flow method, which is often used to monitor chemical or enzymatic reactions or to increase sensitivity [21–23]. However, the stop-flow method can lead to sample degradation due to excitation with deep UV radiation [18]. In contrast, Frosch et al. have shown that DUV-RRS can be used in an aqueous mobile phase and that an extended exposure time can improve sensitivity, while the analyte is protected from degradation due to the continuous flow [24].

The detector developed in this study is intended to offer a variety of applications for pharmaceutical quality control. Therefore, three pharmaceuticals were selected as test substances, each representing standard therapeutic strategies for different medical indications. Epirubicin, a classic cytostatic agent, is used in a wide range of indications such as breast carcinoma, small cell lung carcinoma, advanced ovarian carcinoma and many others. The pharmaceutical product for the application is prepared right before administration in the hospital pharmacy. Further test substances are Metformin and Naproxen. Metformin is the first-line treatment for type 2 diabetes mellitus [25] and Naproxen is a nonsteroidal anti-inflammatory drug (NSAID) against moderate pain, fever, and inflammatory disease. Both are among the most prescribed medications in the United States [26].

In this study, DUV-RRS was coupled with HPLC to identify pharmaceutical substances. The self-built optical device was equipped with an economically efficient and sample-friendly laser, emitting a low average output power of 0.5 mW. However, to achieve a sufficient Raman signal intensity, a low-flow method was developed to extend the exposure time of the analytes after chromatographic separation. In addition to the assignment of Raman signals for the identification of the three test substances naproxen, metformin, and epirubicin, the LODs and LOQs were determined using signal-to-noise ratio (SNR) and partial least square (PLS) analysis.

2. Experimental section

2.1. Chemicals and samples

All chemicals were used without further purification. Naproxen sodium was sourced from BLD Pharmatech GmbH (Kaiserslautern, Germany). Epirubicin hydrochloride (CRS) was obtained from EDQM (Strasbourg, France) and metformin hydrochloride was acquired from Auro Laboratories Limited (Mumbai, India). Sodium dihydrogen phosphate monohydrate was supplied by AppliChem GmbH (Darmstadt, Germany) and sodium dodecyl sulfate (SDS) was obtained from Caesar & Loretz GmbH (Hilden, Germany).

2.2. Sample and mobile phase preparation

Water in HPLC gradient grade was purchased from Fisher Scientific (Geel, Belgium). Acetonitrile as well as methanol were provided by VWR Chemicals (Langenfeld, Germany). These solvents were used for buffer preparation, mobile phase and sample preparation. To adjust the pH of the buffers (phosphate buffer, SDS buffer), phosphoric acid ≥85 % (SIGMA-ALDRICH CHEMIE GmbH, Steinheim, Germany) and sodium hydroxide 1 M (Fisher Scientific, Geel, Belgium) were used.

2.3. HPLC-coupled deep UV resonance Raman detector

The HPLC system (Dionex, Sunnyvale, California, USA) consisted of a quaternary pump (P 580 A), an autosampler (ASI-100), a column oven (STH 585), and a UV detector (UVD 340U). Chromatographic separation was achieved using a Zorbax SB-C18 4.6 × 150 mm, 5 µm (Agilent Technologies, Santa Clara, USA) column as stationary phase. The experimental setup of the HPLC-coupled DUV-RRS is depicted in Fig. 1.

For the DUV-RRS, the laser model NeCu 70-248SL, manufactured by Photon Systems Inc. (Covina, California, USA), was used. The pulsed metal vapor hollow cathode laser provides an emission wavelength of 248.6 nm. A 4-bounce edge filter was used as a plasma line filter for a clean laser profile. A commercial square quartz flow cell with a volume of 16 µL (inner dimensions: 1.5 × 1,5 × 7.1 mm) from a fluorescence detector (RF 2000 Fluorescence Detector, Dionex, Sunnyvale, California, USA) was used as flow cell. The laser beam was adjusted by two excimer laser mirrors (Product No.: 47–985, Edmund Optics, Barrington, New Jersey, USA) and focused into the flow cell using a 1/2″ quartz lens with 20 mm focal length (Product No.: LA4647-UV-ML, Thorlabs, Newton, New Jersey, USA). The Raman scattered light was collected over 90° using a 1" quartz lens (Product No.: LA4052-UV, Thorlabs, Newton, New Jersey, USA). Laser light and Raman scattered light were coupled in and out from the two shorter sides of the flow cell with path lengths of 1.5 mm. When coupling the laser, an attempt was made to hit the cell as centrally as possible. It can be assumed that the laser travels about 0.75 mm through the sample to the focal point. The adjustment was carried out manually according to the best possible Raman signal based on the pure mobile phase. The Raman scattered light collected over 90° also covers a distance of about 0.75 mm through the sample before it leaves the flow cell. The Rayleigh scattering was effectively blocked by a long-pass filter (Product No.: LP02-248RS-25, IDEX Health & Science, LLC, Rochester, New York, USA). The Raman scattered light was focused by a further quartz lens (Product No.: LA4148-UV, Thorlabs, USA) into a round-to-linear fiber bundle with a Ø 105 µm core (Product No.: BFL105HS02, Thorlabs, Newton, New Jersey, USA) and guided to the spectrometer for spectral processing. The experimental setup utilized the Kymera 328i-B2 spectrometer manufactured by Andor Technology/Oxford Instruments plc (UK). For measurements, a high-resolution diffraction grating optimized for the UV range with 2400 lines/mm and enhanced diffraction efficiency at 300 nm was chosen. To detect Raman scattered light, the iDus DV420A-BU2 charge-coupled device (CCD) camera, from Andor Technology/Oxford Instruments plc (Belfast, UK), was equipped. For beam guidance, components from the SYS40

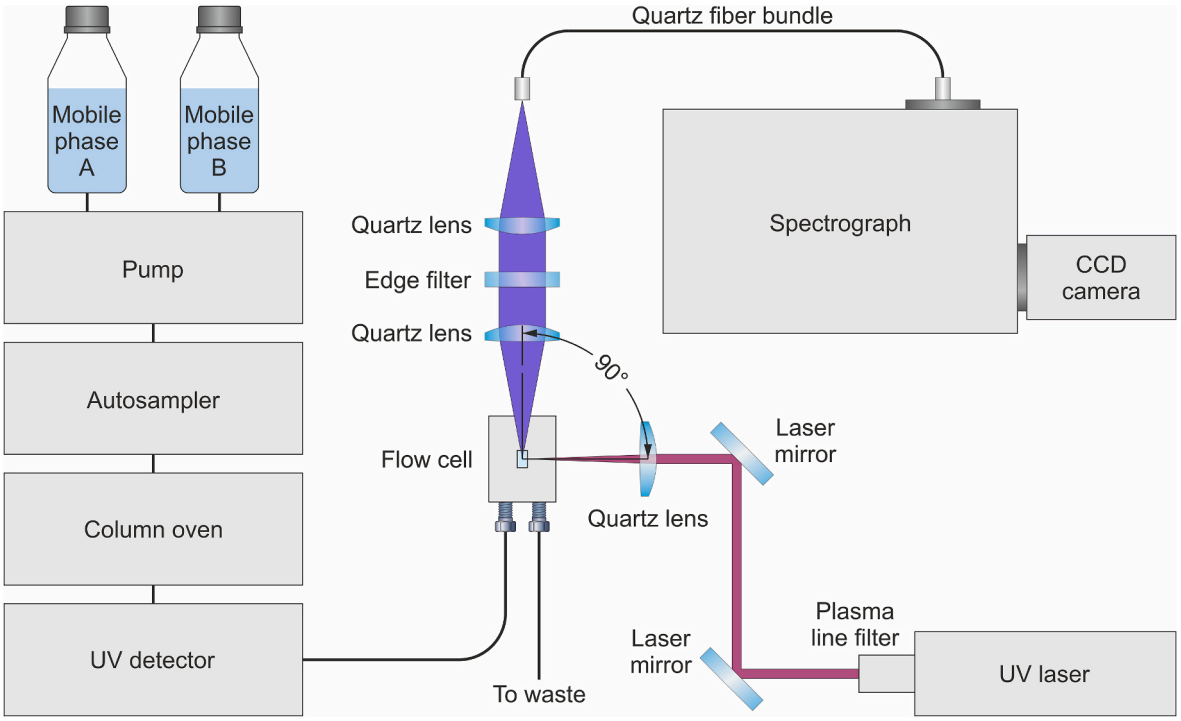

Fig. 1. Experimental setup of HPLC-coupled DUV-RRS as used in this study.

series by OWIS GmbH (Staufen, Germany) were used.

2.4. Acquisition of Raman spectra

To generate the Raman spectra, the laser was configured using the DUV Laser. Ink software from Photon Systems Inc. (Covina, California, USA) to operate at a pulse energy of 25 μJ and a maximum frequency of 20 Hz. These settings result in a power output of 0.5 mJ s^{-1}, which corresponds to an average power of 0.5 mW. Under these conditions, the laser showed sufficient power stability for reproducible measurement of the chromatographic peaks. However, measurements at the maximum pulse energy of approx. 35 μJ led to a drop in laser power of over 15 % in the first 1–2 min. For data acquisition, 12 spectra were accumulated with an exposure time of 5 s each. Therefore, the total measurement time amounted to 60 s. The CCD camera was operated using the software Andor SOLIS 4.32.30091.0 (Andor Technology/Oxford Instruments plc, Belfast, UK). The final data analysis was performed using OriginLab software (Origin 2021b, OriginLab Corporation, Northampton, Massachusetts USA).

2.5. Data processing and signal-to-noise ratio determination

The OriginLab software was utilized for the visualization and processing of the Raman spectra. First, the spectra were cropped to a range of 500–2000 cm^{-1} and then a baseline correction was performed using the "asymmetric least squares" algorithm. The Raman spectra were smoothed using a Savitzky-Golay filter (filter width of 6/polygon degree of 2). Subsequently, the strongest Raman signal assignable to each analyte was subjected to a second baseline correction using the same method as described above. The reason for the second baseline correction can be seen in Fig. 3. The baselines under the Raman signals of the APIs are strongly influenced by the Raman signals of the mobile phase. A further baseline correction provides corrected signal intensities of the analytes. However, a background subtraction according to Dijkstra et al. [18] was not carried out in this study. The S/N was calculated to determine the detection limit of the Raman signals of the three test substances. This involved dividing the signal height of a specific band (signal) by the standard deviation of a spectral region where no Raman signals were expected (noise). For the determination of the noise 24 data points in a row were selected between 1950 and 2050 cm^{-1}. The process of determining the S/N ratio was performed in triplicate to ensure reproducibility, robustness and reliability.

Furthermore, the measured spectra were analyzed in a PLS regression. In this procedure the unscaled spectra of each substance were utilized for the calculation of the concentration of the respective solutions. After leave one out cross validation, the cross validated predictions and loadings were analyzed.

3. Results and discussion

3.1. Method development for low-flow detection

Due to the use of a low laser power of 0.5 mW, online measurements after chromatographic separation with acquisition rates of 0.5–1 s were not successful. For this reason, a low-flow method was developed to significantly extend the sample dwell time in the flow cell. This allowed considerably increased exposure times of the analyte molecules. Consequently, the Raman signals of the analytes could be recorded after chromatographic separation.

The low-flow method was separately developed for each test substance. In all approaches, the flow rate was reduced to 0.05 mL min^{-1} while the highest concentration of the analyte occurred in the flow cell of the DUV-RRS. The HPLC methods used are summarized in Table 1.

In order to determine the most appropriate time to reduce the flow rate, the retention times determined by the UV detector of the HPLC were used as a benchmark. The Raman detector connected after the UV detector shows a time offset of a few seconds. At this point, various time points were systematically tested at which the flow rate was reduced to 0.05 mL min^{-1}. To monitor the concentration of the analytes in the Raman flow cell, Raman spectra were continuously recorded during low-flow operation. Due to low laser power and short exposure time, no Raman signals of the analyte were observed. Instead, the attenuation of the Raman signal from the mobile phase was monitored to determine the presence of the analyte within the flow cell. The attenuation is

Table 1
Chromatographic conditions for low-flow UV resonance Raman detection of naproxen sodium, metformin hydrochloride, and epirubicin hydrochloride.

	Naproxen sodium	Metformin hydrochloride	Epirubicin hydrochloride
Column	Zorbax SB-C18 4.6 × 150 mm, 5 µm (Agilent Technologies)		
Mobile phase	H_2O + $Na_2HPO_4 \cdot 12\ H_2O$ (30 %), B: Acetonitrile (70 %)	A: H_2O + $Na_2HPO_4 \cdot 12\ H_2O$ (50 %), B: Methanol (50 %)	A: H_2O + SDS + H_3PO_4 (50 %), B: Acetonitrile (50 %)
Flow rate	1.0 mL min^{-1}	0.5 mL min^{-1}	0.5 mL min^{-1}
Low-flow rate	0.05 mL min^{-1}		
Injection volume	20 µL		
Concentration	10, 20, 30, 40, 50, 75, 100, 150, 200 µg mL^{-1}		
Mass on column	0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 3.0, 4.0 µg		
UV detection	250 nm		

influenced by two main factors. Primarily, the analyte absorbs a fraction of the laser light, reducing the light available to excite the mobile phase. Secondly, the analyte also absorbs the Raman signals that originate from the mobile phase. Since the attenuation of the Raman signal from the mobile phase depends on the concentration of the analyte, the time at which the analyte reaches its highest concentration in the flow cell can be accurately determined. By integrating the entire Raman spectra and plotting them against time, absorbance chromatograms could be generated using DUV-RRS. Fig. 2 illustrates two chromatograms of naproxen, which were recorded in low-flow operation by the UV and Raman detector. Since the Raman detector is placed behind the UV detector, the time offset of the peak maximum can be observed in the chromatogram. A few seconds after naproxen has passed the UV detector, the flow rate is reduced from 0.5 to 0.05 mL min^{-1}, which increases the offset significantly. While the maximum concentration of the analyte slowly moves through the Raman detector, only the lower concentrated rear part of the substance peak is observed in the UV

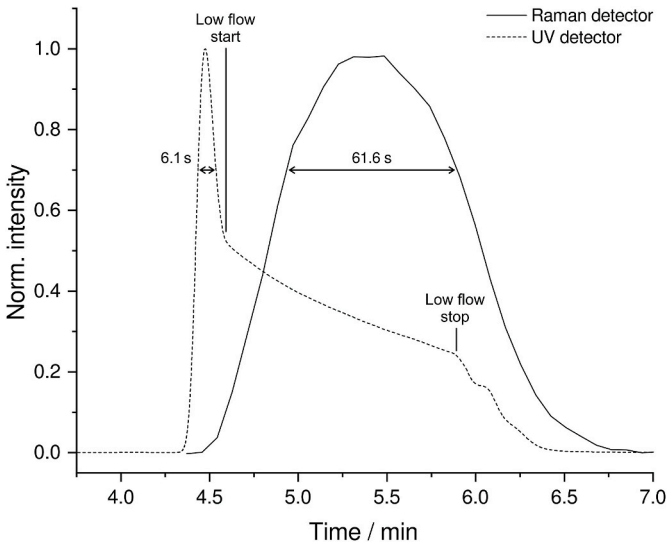

Fig. 2. Normalized low-flow absorbance chromatogram of Naproxen sodium (4 µg mass on column) recorded by the Raman detector (solid line, excitation wavelength at 248.6 nm) and the UV detector (dashed line, absorbance wavelength at 250 nm). To obtain the absorbance chromatogram of the Raman detector, the baseline of each spectrum between 312 and 4463 rel. cm^{-1}, respectively 250.5 and 279.6 nm, was first subtracted. Subsequently, the integrated areas under the spectra were plotted against time (adjacent-averaging, 4 points smoothed). The available measuring time for a Raman measurement is 6.1 s for an online measurement and 61.6 s for a low-flow measurement at a comparable peak concentration ≥70 %.

detector.

For the Raman analysis, a measurement time of approx. 60 s has proven to be a good compromise between high signal intensity and reproducibility. Investigations on laser stability have shown that the pulsed laser used emits a constant power at 20 Hz at maximum intensity ≥ 98 % over a period of approx. 180 s. After this operating time, the laser requires a regeneration cycle of 120 s at low pulse frequency of 7 Hz to be able to emit full power again. With this in mind, it was decided that the measurement should not take longer than 60 s in order to extend the lifetime of the laser. To record the Raman spectra, 12 spectra with an exposure time of 5 s were accumulated over the chromatographic peak maximum. Assuming a readout time of the CCD camera of 0.133 s per spectrum, the total measurement time was 61.6 s, covering a peak intensity between 70 % and 100 %. At a comparable peak concentration, the peak width using a flow rate of 0.5 mL min^{-1} is 6.1 s. Consequently, the measuring time can be increased by a factor of 10 applying low-flow operation and is therefore proportional to the flow rate reduction.

3.2. Sample degradation considerations

As commonly known, the use of UV lasers can lead to degradation of analytes [27,28]. UV radiation can trigger chemical reactions that result in the decomposition of the analytes, potentially leading to inaccurate measurements [18]. The risk of degradation increases with the applied laser power and the exposure time of the sample. In this study, the laser power was reduced at the expense of an increased exposure time.

However, unlike the stop-flow method, in the low-flow method the analyte molecules have only brief contact with the laser light due to the ongoing flow. This prevents prolonged exposure time and minimizes the risk of degradation. At a reduced flow rate of 0.05 mL min^{-1}, the flow cell with a capacity of 16 µL is completely flushed approx. 3.2 times within the 61.6 s measurement duration. This means that the entire content of the flow cell was exchanged every 19.2 s. Nevertheless, the relevant exposure time, which may lead to a degradation of the analyte, mainly refers to the dwell time in the laser focus.

Using a beam diameter of 3 mm and a focusing lens with a focal length of 20 mm, a theoretical focus diameter of approx. 42 µm can be calculated for the applied laser. The thickness of the flow cell is 1.5 mm. If the laser focus is adjusted to the center of the flow cell, the diameter of the laser beam at the point of entry into the analyte can be estimated to 113 µm. To determine the volume excited, the diameter of the laser beam at the cell entrance and the thickness of flow cell were used, which results in a cylindrical volume of 10 nL. This volume is exchanged approximately 83 times per second at a flow rate of 0.05 mL min^{-1}. At a pulse frequency of 20 Hz and a pulse width of 40 µs, each analyte molecule is excited with a maximum of one laser pulse. In addition to the short contact time of analyte and laser radiation, the low risk of photodegradation can be explained by the combination of low laser power, relatively long pulse duration avoiding a high peak power, movement of the analyte due to the flow and the solvent environment for heat dissipation. All these factors underline the sample-friendly nature of this detector.

3.3. Raman analysis

The spectra presented in Fig. 3 were obtained using the low-flow method for naproxen sodium, metformin hydrochloride, and epirubicin hydrochloride, with each substance at an absolute amount of 4 µg on column. The overview spectrum in Fig. 3A shows that the Raman signals of the mobile phase are strongly pronounced. Nevertheless, distinct Raman signals could be assigned to the analytes. As shown in Fig. 3B, the signals at wavenumbers 1156 cm^{-1}, 1581 cm^{-1}, and 1628 cm^{-1} were attributed to naproxen. In Fig. 3C, the signals at 601 cm^{-1}, 728 cm^{-1}, 925 cm^{-1}, and 1287 cm^{-1} were assigned to metformin and the Raman signals at 1076 cm^{-1}, 1564 cm^{-1}, and 1637 cm^{-1} could be attributed to epirubicin, as shown in Fig. 3D.

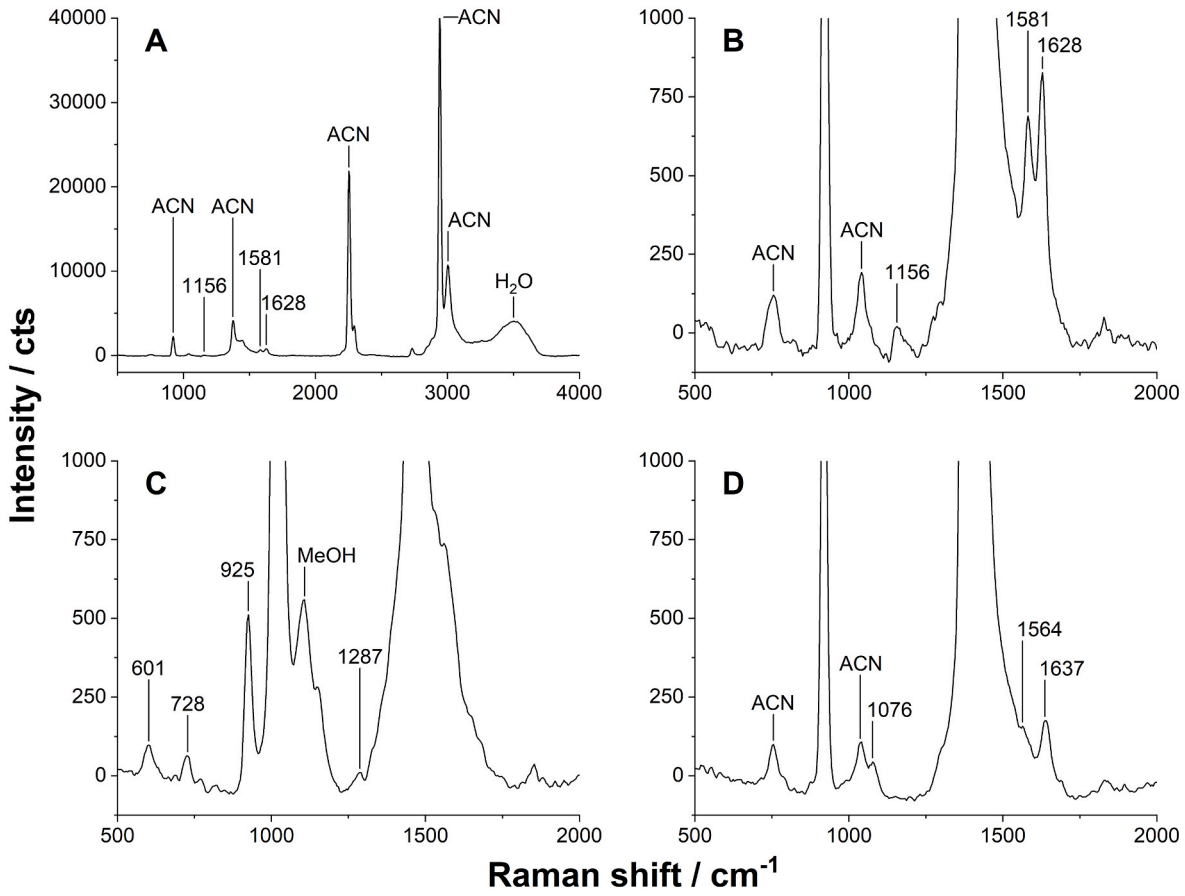

Fig. 3. HPLC-DUV-RRS spectra of naproxen (A, B), metformin (C), and epirubicin (D). Spectrum A displays the entire spectral range, highlighting the dominance of solvent bands from acetonitrile (ACN) and water (H_2O). The Raman signals ascribed to naproxen were marked with their corresponding wavenumbers. Spectra B-D show the Raman fingerprint region as a zoomed-in section for each analyte. Each substance was injected at a volume of 20 µL with a concentration of 200 µg mL^{-1} (4 µg on column). For the Raman spectra, data were collected in low-flow mode, accumulating 12 spectra with an exposure time of 5 s per spectrum over the peak maximum.

In a previous study it was concluded that the application of a stop-flow method to increase the sensitivity of a UV-RRS-HPLC coupling did not provide any advantage [18]. Contrary to this assumption, we were able to show that the use of a low-flow method provides a significant improvement in sensitivity, as online measurements yielded no Raman signals even at the highest concentration of 4 µg on the column. Despite the improved sensitivity, the available spectral range is highly limited due to the interference of the dominant Raman signals of the mobile phase. However, unlike the analyte, the mobile phase is not resonantly excited. This unilateral amplification allows the Raman signals of the analytes to be distinguished from the solvent bands of the mobile phase. Raman signals could be successfully generated and assigned for each specified concentration according to Table 1 with an absolute mass on column between 0.2 and 4 µg. The limit of detection (LOD) and limit of quantification (LOQ) were determined for each test substance using the SNR of the most dominant Raman transition. The results of the linear regression are shown in Fig. 4 and summarized in Table 2.

According to literature data, the LOQ of epirubicin hydrochloride using HPLC-UV detection at 233 nm was determined as 6.3 ng on the column [29]. Thus, the Raman detector presented here is 300 times less sensitive compared to an UV absorbance method. For metformin hydrochloride, a LOQ of 50 ng on the column was found using HPLC-UV, which is only 13 times below the LOQ of the Raman detector [30]. For naproxen sodium the LOQ using HPLC-UV detection was determined to be 0.3 ng on the column, which is approx. 2000 times lower than the LOQ of the Raman detector [31]. The comparison of the two methods

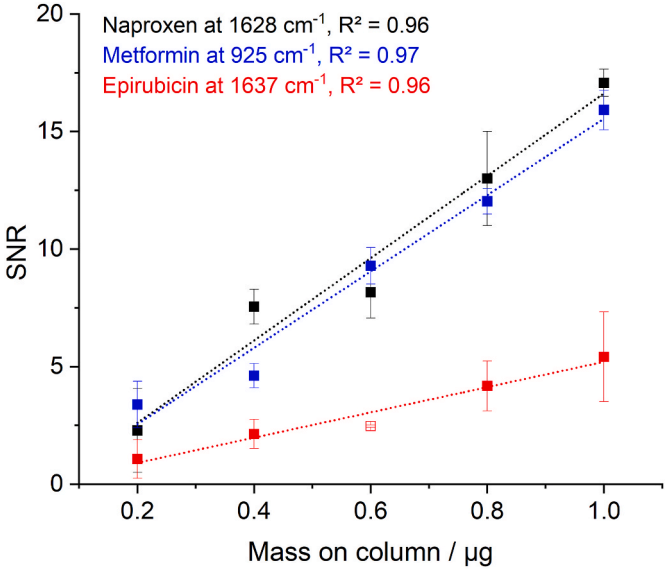

Fig. 4. Linear regressions of the signal-to-noise ratios of the most prominent Raman transitions of naproxen at 1628 cm^{-1}, metformin at 925 cm^{-1}, and epirubicin at 1637 cm^{-1} against the injected mass on the chromatographic column.

Table 2
LOD and LOQ of naproxen sodium, metformin hydrochloride, and epirubicin hydrochloride for the most pronounced Raman transition after chromatographic separation using the low-flow method.

	Naproxen sodium	Metformin hydrochloride	Epirubicin hydrochloride
Raman signal	1628 cm^{-1}	925 cm^{-1}	1637 cm^{-1}
R^2	0.96	0.97	0.96
Linear regression (y = SNR, x = Mass on column)	y = 17.513x – 0.8934	y = 16.247x – 0.6995	y = 5.3689x – 0.1657
LOD (SNR = 3.3) Mass on column	0.24 µg	0.25 µg	0.65 µg
LOQ (SNR = 10) Mass on column	0.62 µg	0.66 µg	1.89 µg

illustrates that the UV detector is more sensitive for analyte quantification as compared to the Raman detector.

However, compared to previous work on the integration of Raman spectroscopy in HPLC using organic fractions in the mobile phase, our work showed similar and, in some cases, significantly improved detection limits while utilizing lower laser power. In a non-resonant setup by Dijksta et al. using a liquid core waveguide and an excitation wavelength of 514.5 nm, LODs of 5 and 250 µg mass on the column were found depending on the injected substance [32]. In comparison, our detector has a 7- to 1000-fold lower LOD. Another work by the same author presented an HPLC-UV-RRS coupling using an excitation wavelength of 244 nm and a laser power of 70 mW [18]. The LODs were determined between 0.175 and 0.70 µg mass on the column. Despite using low laser power, our setup shows a similar detection limit between 0.24 and 0.65 µg on column using a 30-fold higher detection time. However, a direct comparison of the overall performance of the Raman systems developed in the studies is not possible, as completely different laser technologies and spectrometers were used. In addition, different substances with different extinction coefficients were investigated.

3.4. Applicability of UV resonance Raman spectroscopy depending on the substance

The applicability of the HPLC-DUV-RRS coupling is highly sample-dependent. On the one hand, molecules with a chromophoric system are required that absorb at the laser excitation wavelength, in our case at 248.6 nm. As already described in the experimental setup, the Raman scattered light has to travel a path length of about 0.75 mm through the sample before it leaves the flow cell. A part of the Raman scattered light is reabsorbed by the analyte itself, which leads to an attenuation of the Raman signal. Ideally, the analyte shows high absorption at the excitation wavelength, but low absorption in the spectral range of the Raman signals. Fig. 5 shows the absorbance spectra of the three measured substances naproxen, metformin and epirubicin. The spectra were normalized to each other according to the respective molar extinction coefficients at 248.6 nm.

Although epirubicin showed the strongest absorbance at the laser wavelength, the LOD and LOQ values were the highest. In contrast, naproxen shows the lowest absorbance upon excitation, but has the lowest LOD and LOQ values. This contradiction can be explained if the reabsorbance of the Raman signals is included in the evaluation. For this purpose, ratios were formed between the absorbance of the laser wavelength at 248.6 nm and the strongest Raman transitions, which were used to determine the LODs and LOQs. Naproxen shows the highest ratio with a factor of 4.55, followed by metformin at 3.24 and epirubicin at 1.20. The higher the ratios, the more appropriate a substance is for analysis with deep UV Raman spectroscopy. In this way, predictions regarding suitability for this method can already be made on the basis of the UV spectra. However, this is only a first orientation, as the chromatographic method and the selected solvent of the mobile phase also have an influence on the sensitivity.

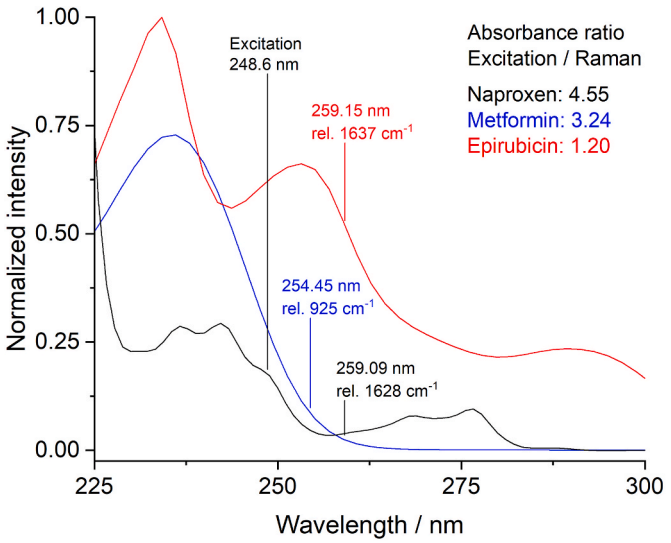

Fig. 5. UV absorbance spectra of naproxen, metformin and epirubicin, measured with the UV detector of the HPLC after chromatographic separation. The spectra are normalized according to the molar extinction coefficients at the laser excitation wavelength at 248.6 nm, which were determined using a 5-point calibration line (0.2, 0.4, 0.6, 0.8, and 1.0 µg on the column). The dominant Raman signals of each analyte are indicated in the UV spectra. The absorbance ratios between the absorbance at the excitation wavelength and the dominant Raman signal were determined for each substance.

3.5. PLS analysis for validation of the low-flow method

In this study, a PLS model was employed to validate the developed low-flow method for each analyte. The experiments revealed that the analytes not only absorb the excitation light, but also the Raman scattered light of the mobile phase. The Raman detector therefore also features the properties of a UV absorbance detector and can in principle be used for quantification. The PLS regressions were calculated across the entire range of wavelengths of all spectra via the algorithms of the scikit-learn 1.3.0 library in Python 3.10. The raw data were not pre-processed for the analysis. For determining the optimal number of latent structures, the mean squared error was reduced using a leave-one-out cross-validation. All presentations of the predictions of the PLS models within this manuscript show the cross-validated results so that the predictability of new data points can be estimated. Fig. 6A shows the measured vs. the predicted mass values of the calibration curve for Naproxen. Fig. 6B presents the relative difference between the spectra of the highest and the lowest concentration of Naproxen over the wavenumber. Below in red lines the loadings of the first and second latent structures are presented. This can be addressed as the contribution of each wavelength to the information content of the spectra regarding the change in the concentration. In this presentation the influence of the absorbance of the Raman scattered light of the mobile phase on the PLS results is evident. The relative difference of the intensity shows clear peaks at wavenumbers correlating with peaks of the mobile phase. Nevertheless, additional pattern in ranges of the spectrum of Naproxen indicates that not only the absorbance effects of the mobile phase contribute to the results. The coefficients of determination of the cross validated results for all analytes can be found in Table 3, each displaying an R^2 value exceeding 0.98, indicating remarkable precision of the method. This result ensures that the low-flow procedure can be set up reproducible to measure the exact peak position of the substance within the detector. Moreover, the LODs for the PLS model were determined according to the method published by Ortiz et al. [33]. Compared to the LODs of the Raman signals, the LODs of the PLS model are lower by a

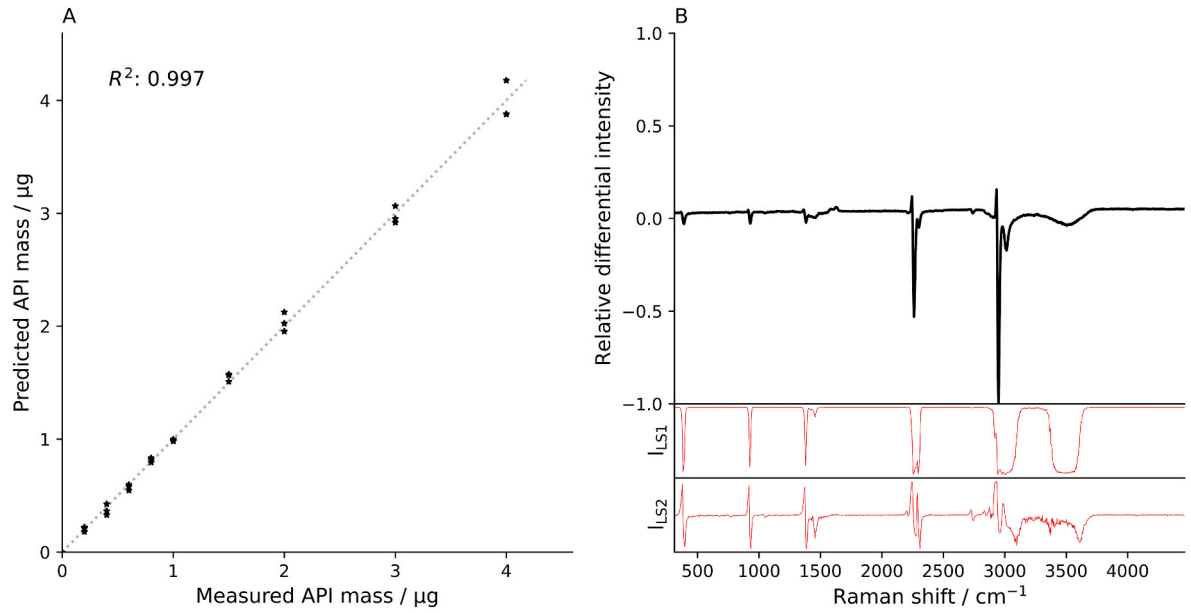

Fig. 6. A. Measured vs. predicted mass of Naproxen, B. Relative differential intensity between the maximum and minimum concentration of the substance (black) and loadings of the first (I_{LS1}) and the second (I_{LS2}) latent structure of the model (red).

Table 3
Determination coefficients of naproxen sodium, metformin hydrochloride, and epirubicin hydrochloride.

	Naproxen sodium	Metformin hydrochloride	Epirubicin hydrochloride
R^2	0.997	0.98	0.996
LOD Mass on column	0,0144 µg	0,0141 µg	0,0141 µg

factor of 15–45. However, these values should only be considered as rough estimates, as no additional measurements for the PLS model were taken in the proximity of the LOD, as this was out of the scope of this study. The UV detector of the HPLC is still preferred for quantification in this setup, as it provides a significantly higher sensitivity and records more data points across the entire chromatographic peak due to faster readout.

One challenge of the low-flow method is that the timing of the flow reduction needs to be adjusted at regular intervals. This is mainly due to retention time shifts, which can be caused, for example, by column ageing, changing pH values of the mobile phase or temperature changes. However, due to the positioning of the Raman detector behind the UV detector of the HPLC, the chromatographic offset can be utilized to avoid this issue. Assuming that the capillary volume between the two detectors is known, a trigger signal can be transmitted from the UV detector to activate the low-flow mode on the one hand and to start the Raman measurement on the other. The flow reduction occurs approximately 6 s after the peak maximum has passed the UV detector and the Raman measurement starts another 21 s later (Fig. 2). With appropriate software development, the low-flow method can therefore be automated in future work.

A comparable chromatographic technique that is already commercially available uses the heart-cutting and peak-parking concept. The idea of cutting and subsequently reanalyzing peaks proves valuable in the context of two-dimensional liquid chromatography (2D-LC). In this approach, an area of interest with one or more peaks can be cut out and parked for a second chromatographic separation. This is especially beneficial when dealing with complex mixtures where different components exhibit similar behaviors which can prevent a complete separation in the first dimension [34,35]. Another application involves parked peaks for subsequent analysis using a Raman detector. Heart-cutting the peaks allows analyzing samples offline after separation, providing both qualitative and quantitative information's based on the obtained results [36]. Using this method, it would not be necessary to alter the flow rate during the chromatographic run. Instead, individual peaks could be parked and subsequently analyzed with the low-flow technique using the Raman detector.

4. Conclusion

The HPLC-coupled DUV-RRS developed in this study proved to be a precise, sensitive and effective tool for pharmaceutical analysis. However, there is a limitation for substances that do not exhibit an absorption band at the excitation wavelength of 248.6 nm, as no resonance Raman effect is induced. Despite a low average laser power of 0.5 mW, we were able to successfully generate and assign Raman signals for naproxen, metformin, and epirubicin by combining it with a low-flow chromatographic method. Multivariate data analysis demonstrated the accuracy of the low-flow method and that the peak position for the Raman measurement was reproducible. The combination of DUV-RRS with peak-parking concepts may contribute to the automation of the system.

The LOD was determined to an absolute mass between 0.24 µg and 0.65 µg, which was loaded onto the column of the HPLC. Compared to previous studies on coupling UV resonance Raman spectroscopy with HPLC using organic solvents in the mobile phase, the detection limit was in a similar range. A further improvement in sensitivity may be achieved by utilizing an LCW. However, coupling DUV-RRS to HPLC using an LCW has not yet been demonstrated and will be investigated in future work.

Obviously, the increase in laser power would also contribute to an improvement in signal intensity. Technological progress will certainly produce new laser sources. In particular, developments in deep UV LEDs have been very successful in recent years and provide the potential for innovative, low-cost Raman lasers. This would facilitate the development of DUV-RRS, especially with regard to affordable detectors to be used in HPLC.

In summary, the developed HPLC-DUV-RRS coupling offers a valuable and sustainable approach for a wide range of substances in

pharmaceutical analysis, as many pharmaceuticals exhibit absorption at 248.6 nm. By overcoming the limitations of conventional Raman spectroscopy, this method contributes to the extension of analytical techniques in the pharmaceutical industry. As a detector with the capability to identify APIs, it may provide a cost-effective alternative to mass spectrometric analysis for certain applications.

Notes

The authors declare no competing financial interest.

CRediT authorship contribution statement

Philipp Siegmund: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Stefan Klinken:** Writing – original draft, Visualization, Formal analysis, Conceptualization. **Michael C. Hacker:** Writing – review & editing, Supervision. **Jörg Breitkreutz:** Writing – review & editing, Supervision. **Björn Fischer:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Bjoern Fischer reports financial support was provided by Federal Ministry for Economic Affairs and Climate Action. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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