Application Note LCMS-115

Automated Quality Control of Active Pharmaceutical Ingredients (API) by LC-UV-ion trap MS using Compass Open Access: a Case Study of Gamma-Irradiated Oxybutynin

Abstract

This case study was initiated after detection of an unknown degradation product in a gamma-irradiated sample of the anticholinergic drug oxybutynin hydrochloride during routine drug substance QC. The sample was qualified as being non-compliant with the specifications of the Ph. Eur. monograph 01/2005:1354 [1] which defines a limit of 0.1% for unspecified impurities. For the characterization of the oxybutynin degradation products by mass spectrometry an LC-UV-ion trap MS^n method was developed. Using Bruker software such as MetaboliteTools (for advanced differential peak detection) and FragmentationExplorer (for interactive MS/MS spectrum interpretation) the impurities were identified as phenylcyclohexyl glycolic acid and cyclohexyl phenyl ketone (CPK). To the best of our knowledge this is the first time CPK has been described as irradiation product of oxybutynin.

For a comprehensive quality control of oxybutynin samples an automated workflow based on Bruker Compass OpenAccess QC software was established. The verification of the drug substance identity is based on its molecular formula and MS^n spectral library matching; the identity of the impurities is verified by UV and MS spectra matching as well. The purity of the oxybutynin samples is determined by UV detection at 210 and 254 nm.

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gamma sterilization	MetaboliteTools
API	

The results of this case study on gamma-irradiated oxybutynin clearly illustrate the additional value and complementary character of ion trap mass spectrometry in addition to UV detection for drug substance QC.

Introduction

The pharmaceutical quality of active pharmaceutical ingredients (API) and other drug substances is controlled by specific and general monographs in pharmacopoeia (Ph. Eur. [1], USP [2]). Methods to test identity, purity and content are specified. Impurities are anticipated to originate from the source material, drug synthesis and drug purification processes, or to be degradation products. Analysis of non-volatile organic impurities is mainly performed using HPLC methods with UV detection. This approach is considered to be sufficiently specific and sensitive for known impurities or related substances possessing a similar chromophore as the parent compound with comparable molar extinction coefficient. Verification of the identity of known impurities is mainly done on the basis of (relative) chromatographic retention times. The number of monographs in Ph. Eur. and USP that prescribe HPLC or GC with mass spectrometric detection, enabling identification of impurities by mass and MS^n, is still very limited. However, there is an ongoing effort to revise and adjust "old" monographs and to introduce new methods of analysis.

Gamma irradiation can be applied to decontaminate and/or sterilize a wide range of medical devices and pharmaceutical components. Many pharmaceutical products are degraded to some extent upon treatment with high-energy radiation. Drugs in solid state, including pure active pharmaceutical ingredients, are more stable towards radiation than drugs in aqueous media.

The EU scientific guideline concerning the use of ionizing radiation in the manufacture of medicinal products requires not just validation with regard to the purpose of irradiation (usually sterilization), but also validation with regard to the quality of the product [3]. Chapter 4.3 of this guideline states that "information should be given about the formation of radiolysis products or other degradation or interaction products. Whenever possible, the radiolysis products should be identified". A manufacturer of irradiated products or those using irradiated drug substances in the manufacturing process will have to perform studies to determine the extent of radiation stability. It is expected that impurity profiling on the basis of the existing compendia monographs will usually not suffice to characterize drug substances subjected to ionizing radiation.

A study has been carried out to investigate the effect of gamma radiation on the drug oxybutynin hydrochloride. Oxybutynin is an anticholinergic, spasmolytic drug used in the treatment of urinary incontinence. Administered orally, it shows many side effects, but these can be largely avoided by intravesical treatment. For this application a sterile product is required. The drug substance oxybutynin HCl is described in both, Ph. Eur [1] and USP [2]. Some orally administered products containing oxybutynin are described in USP [2] and BP [4]. The prescribed LC-UV method for the control of impurities is not suited to provide information about the identity of radiation induced degradation products. Therefore, an LC-UV-ion trap MSn (LC-UV-ITMS) method was developed for the identification of oxybutynin degradation products. In addition, an automated workflow based on Compass OpenAccess software was set-up for the quality control of oxybutynin hydrochloride; enabling a push-button solution for drug substance verification and impurity profiling.

Experimental

Samples and sample preparation

Oxybutynin hydrochloride (drug substance, Ph. Eur. quality) was stored in a climate room at 25 °C/60% relative humidity after treatment with gamma radiation ("irradiated sample"), and as such (not irradiated "control sample"). Stock solutions of 4 mg/mL in water were prepared from oxybutynin control and the irradiated sample. For the direct infusion experiment stock solutions were diluted to obtain a final concentration of 2 µg/mL in H_2O/ACN (50/50), 0.1% HCOOH. For LC analysis aqueous solutions with the following concentrations were prepared: 0.5, 1 and 2 mg/mL. For cyclohexyl phenyl ketone (CPK, Sigma-Aldrich 139211) a stock solution of 4 mg/mL water was made and further diluted to give concentrations of 200, 20 and 2 µg/mL.

For QC analysis using Compass OpenAccess QC, solutions of oxybutynin control and oxybutynin irradiated sample were injected at a concentration of 1 mg/mL. Additionally, the 1 mg/mL oxybutynin control was spiked with 0.1% and 1% (w/w) cyclohexyl phenyl ketone and submitted for QC measurements.

Direct infusion experiment

In order to obtain information about molecular ion and fragments of oxybutynin the control sample was analyzed in direct infusion mode using a syringe pump for continuous sample introduction. A solution of 2 µg/mL was infused at a flow rate of 3 µL/min and MS and MS/MS experiments were performed.

UHPLC conditions

The Ultimate 3000 RSLC System (Thermo Scientific) was equipped with an Acquity BEH C8, 1.7 µm, 2.1 x 100 mm column (Waters) and a photo diode array detector (200-350 nm). The column temperature was set at 40°C; injection volume was 5 µL. Solvent A (5 mM ammonium acetate in water) and solvent B (5 mM ammonium acetate in acetonitrile/water (90/10; v/v)) were used for gradient elution at 0.25 mL/min. For gradient details please refer to table 1.

Table 1: HPLC gradient for separation of oxybutynin and its irradiation products .

min	%B
0	20
30	60
35	100
40	100
41	20
44	20

MS conditions

MS analysis was performed using an amaZon SL ion trap mass spectrometer (Bruker Daltonics) equipped with an Apollo II ESI or APCI source. Measurements were carried out in positive and negative ion mode. Tuning was accomplished by Smart Parameter Setting SPS™ for target mass 200 or 350 *m/z* respectively. The scan mode employed was UltraScan (32.500 *m/z* s-1) at a scan range of 50-400 *m/z*. MS^2 and MS^3 spectra were acquired in data-dependent mode.

The LC-MS system was equipped with the MetaboliteTools software (version 2.1, Bruker) for the 1:1 comparison of LC-MS data sets and Compass OpenAccess (version 1.4, Bruker) for automated routine API QC analysis.

Automated workflow for API verification and purity determination

QC measurements were carried out in a fully automated manner using Compass OpenAccess. Sample information was submitted either manually or for a larger number of samples via an Excel csv file. The "Oxybutynin QC method" was selected, samples were placed in the UHPLC autosampler, and the acquisition was started from Compass OpenAccess. Analysis was done using APCI negative mode in the first 8 min and positive APCI for the rest of the chromatographic run. Full scan MS and MS/MS spectra were recorded in data dependent mode to determine the molecular mass and obtain structure information for the API and the impurities present in the sample. After the acquisition, data processing including report generation was done fully automatically. First, the identity of the main compound was verified by comparison of theoretical and experimental molecular weight followed by MS and MS/MS spectra match with a corresponding spectral library. During the second step of the QC workflow, UV traces were integrated to calculate the relative peak area of the impurities and to verify their identity by comparison of UV and MS spectra with the spectral library.

Results and discussion

From a direct infusion experiment using the control sample, MS and MS^2 spectra of oxybutynin were obtained. The MS^2 spectrum was interpreted and annotated using the FragmentationExplorer software tool (Fig. 1).

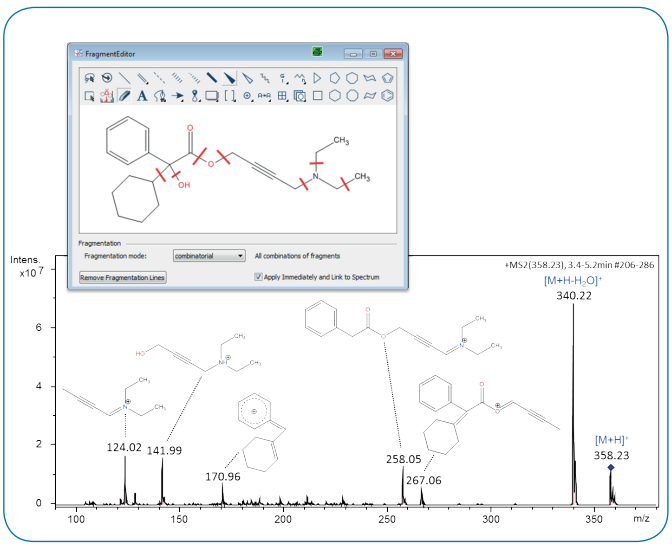


Fig. 1: Structure with potential cleavage sites and MS/MS spectrum of oxybutynin. Annotation with proposed fragment structures was done using the Bruker FragmentationExplorer.

The LC-UV methods for the impurity profiling of active pharmaceutical ingredients described in the monographs often use non-volatile buffers. Such buffers, like potassium phosphate, are typically not compatible with mass spectrometry. Thus, a new eluent system was established using ammonium acetate as additive. The LC gradient was optimized to allow adequate retention and separation of the drug substance and its degradation products. UV detection was performed at two wavelengths, 210 nm as it is used in the monograph method [1], and 254 nm was used to have additional specificity for aromatic (degradation) products. Using the new method, LC-UV analysis at 210 and 254 nm showed two additional peaks in the gamma-irradiated oxybutynin sample (compared to the control sample), eluting at 5.5 and 23.5 min, respectively (Fig. 2). When analyzing the samples by LC-UV-ITMS using electrospray ionization (ESI) in the positive ion mode both irradiation products turned out to have poor ionization efficiency (data not shown). In the ESI negative ion mode an intense peak was detected for the impurity with retention time 5.5 min.

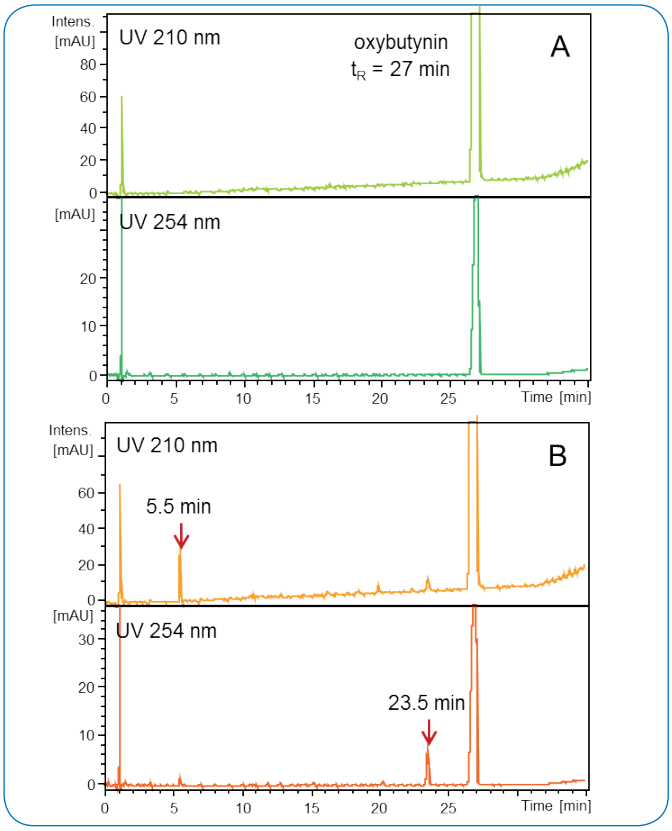


Fig. 2: LC-UV chromatograms of irradiated sample (B) show two additional peaks at 210 and 254 nm in comparison to the oxybutynin control (A).

The Bruker MetaboliteTools software package allows in-depth evaluation of small differences between two LC-MS data sets [5]. The difference calculation is based on the so-called eXpose algorithm. All MS signals which are significantly (typically a factor of 3) higher in a sample compared to a reference will be displayed in the MS difference chromatogram. Fig. 3 shows a comparison of the UV traces at 210 and 254 nm with the MS difference chromatogram calculated by MetaboliteTools for the negative ESI mode.

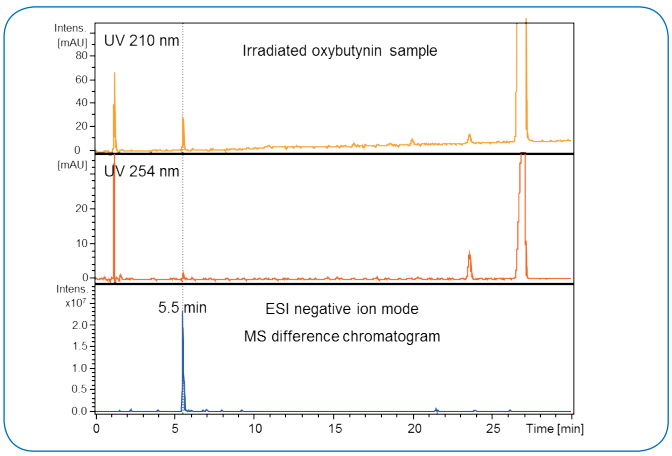


Fig. 3: UV chromatograms of irradiated oxybutynin and MS difference chromatogram calculated by MetaboliteTools in negative ESI mode. The major peak in the MS difference chromatogram corresponds to the UV signal at 5.5 min.

The major MS peak at 5.5 min correlates very well with the UV peak at 210 nm. The corresponding spectra showed an intense MS signal at *m/z* 233 and an MS/MS fragment deriving from the loss of 44 Da (Fig. 4). These data indicate the presence of phenylcyclohexyl glycolic acid or "Impurity D" [1].

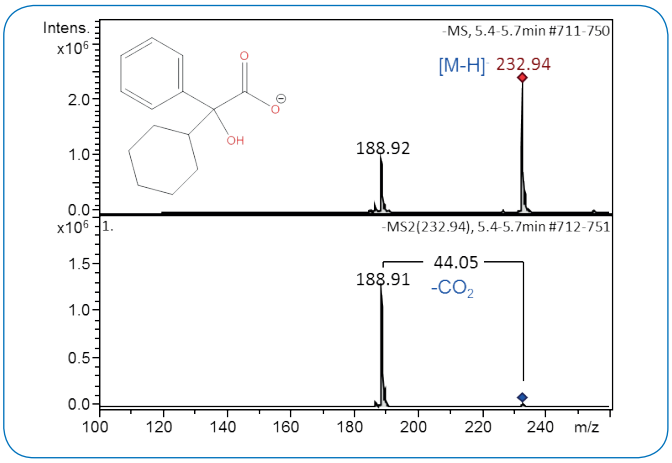


Fig. 4: MS and MS/MS spectrum of irradiation product at 5.5 min in negative ESI mode led to the identification of phenylcyclohexyl glycolic acid (or "Impurity D" [1]).

The second UV active degradation product was detected using atmospheric pressure chemical ionization (APCI) in positive ion mode. In contrast to negative APCI where only one peak was present (data not shown), the positive APCI mode gave multiple signals in the MS difference chromatogram. One of the peaks could be assigned to the UV peak at 23.5 min (Fig. 5).

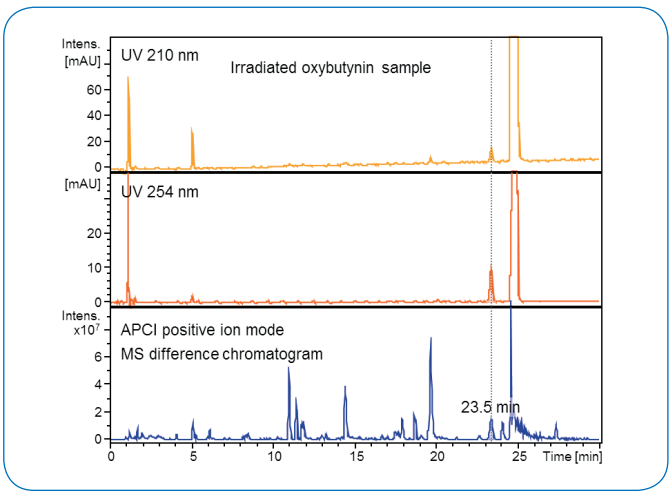


Fig. 5: UV chromatograms of irradiated oxybutynin and MS difference chromatogram in positive APCI mode calculated by MetaboliteTools. The UV peak at 23.5 min can be correlated with one of the peaks in the rather complex MS difference chromatogram.

Based on the comparison of acquired MS and MS^2 spectra with those obtained from a reference standard the impurity was identified as cyclohexyl phenyl ketone (CPK, Fig. 6), which to the best of our current knowledge has not been described as irradiation product of oxybutynin so far.

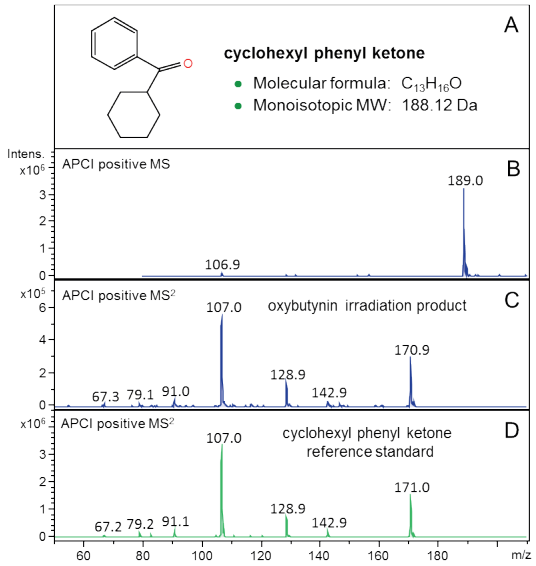


Fig. 6: (A) Structure of cyclohexyl phenyl ketone; (B, C) MS and MS^2 spectrum of oxybutynin irradiation product at t_R = 23.5 min; (D) MS^2 spectrum of cyclohexyl phenyl ketone reference standard.

The identification was finally confirmed by co-elution experiments. For this purpose, the CPK standard was spiked at a concentration of 4 µg/mL into the irradiated oxybutynin sample (1 mg/mL), which led to a clear increase of the peak area of the impurity at 23.5 min in UV and MS chromatogram (extracted ion chromatogram EIC 189 *m/z*; Fig. 7).

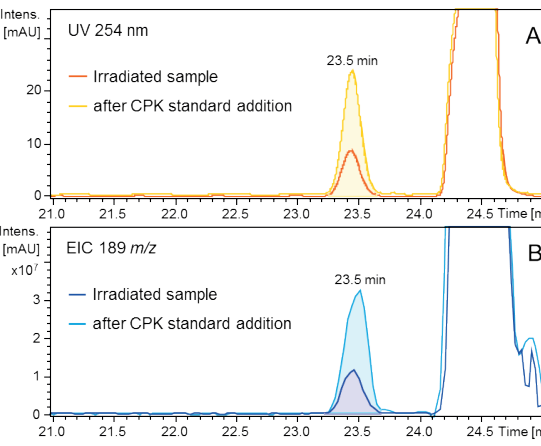


Fig. 7: Addition of cyclohexyl phenyl ketone (CPK, 4 µg/mL)) to the irradiated oxybutynin sample (1 mg/mL). (A) LC-UV chromatogram at 254 nm and (B) EIC at 189 *m/z* before and after addition of CPK standard.

In the positive APCI mode multiple degradation products with very low UV response were observed (Fig. 8A). According to the Ph. Eur these compounds may be disregarded since their UV based relative abundancies are below the limit of 0.05% [1]. Based on the detected $[M+H]^+$ and the MS^n fragmentation pattern, for one of these minor compounds an oxybutynin related structure was proposed (Fig. 8B).

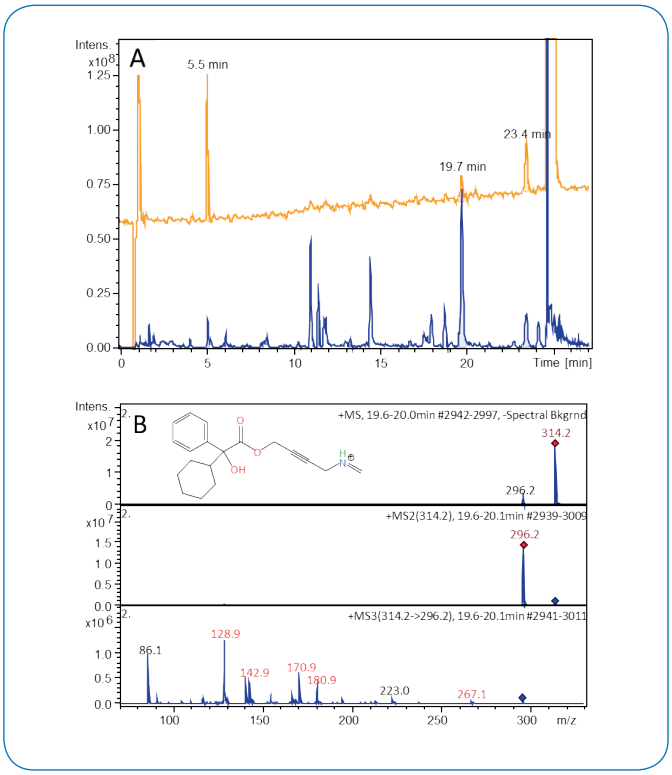


Fig. 8: (A) UV chromatogram at 210 and MS difference chromatogram. "Disregarded compounds": Impurities with a relative intensity < 0.05% at 210 nm, e.g. peak at 19.7 min.
(B) Structure proposal for compound eluting at 19.7 min based on its MS^2 and MS^3 spectrum. Fragment masses marked in red are in common with oxybutynin or cyclohexyl phenyl ketone.

Automated workflow for QC of oxybutynin using Compass OpenAccess

Now the impurities have been characterized, the UV, MS^n spectra and retention time data were used to create a spectral reference library. Using this library an automated workflow was created in the Compass OpenAccess software [6] for the quality control of oxybutynin samples (Fig. 9).

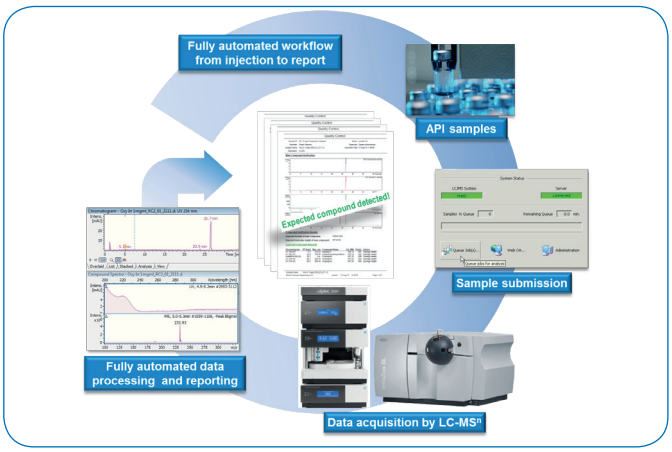


Fig. 9: Schematic presentation of automated QC workflow based on Compass OpenAccess

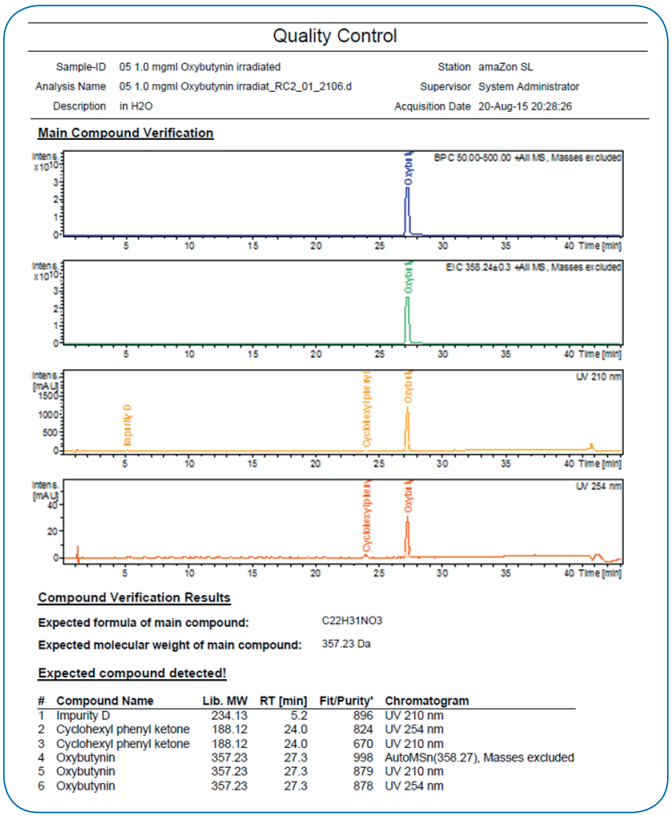
Four different samples were used to evaluate the workflow. Oxybutynin control and irradiated oxybutynin samples were prepared at a concentration of 1 mg/mL. Additionally, the oxybutynin control sample was spiked with 1 and 0.1% (w/w) CPK as the limit for unspecified impurities is 0.1% relative to the drug compound based on UV absorption [1]. The results for the irradiated oxybutynin sample are shown in Fig. 10 and 11.

- Fig. 10 shows the first page of the automatically generated QC report. It includes the base peak chromatogram (BPC), an extracted ion chromatogram (EIC) for oxybutynin and UV traces at 210 and 254 nm. The *m/z* value of the EIC is derived from the molecular formula of oxybutynin ($C_{22}H_{31}NO_3$) which has to be specified in the QC method. Below are the details of the compound verification based on the comparison of theoretical and measured mass with the final result: "Expected compound detected!". The summary of the library search is displayed in the following table. Oxybutynin was confirmed by MS/MS with a Purity' score of 998 (a value of 1000 indicates 100% identity). For additional visualization, measured MS and MS^2 spectra of oxybutynin are displayed together with the corresponding library spectra on the second page of the QC report (data not shown).

- Impurity profiling is based on the relative peak area determined from the UV chromatograms at 210 and 254 nm. Results are given in Fig. 11. Due to clear differences in the extinction coefficients of oxybutynin and its degradation products, different values are obtained from the two wavelengths. For the verification of the identity of the impurities UV, MS and library spectra are shown.

All results of the impurity profiling of the four samples are summarized in table 2. The oxybutynin control sample gave only one peak in the UV and thus had a purity of 100%. In both spiked samples oxybutynin and CPK were detected and verified. The relative abundances of CPK as determined at 210 nm resemble the spiked levels, indicating a (semi-) quantitative relation between oxybutynin and its degradation product at this wavelength.

At 254 nm however, the relative abundances of CPK were 5.4% and 33% respectively due to the higher response of CPK compared to oxybutynin at this wavelength. In the irradiated oxybutynin sample the relative abundance at 210 nm was determined to be 0.13% for CPK, which is in agreement with the initial findings from routine drug QC, and 0.44% for Impurity D. At 254 nm only CPK was detected with a relative abundance of 5.4% reflecting the overestimation of the CPK content at this wavelength.



Quality Control

Sample-ID 05 1.0 mgml Oxybutynin irradiated
Station amaZon SL
Analysis Name 05 1.0 mgml Oxybutynin irradiat_RC2_01_2106.d
Supervisor System Administrator
Description in H2O
Acquisition Date 20-Aug-15 20:28:26

Main Compound Verification

BPC 50.00-500.00 +All MS, Masses excluded
EIC 358.24±0.3 +All MS, Masses excluded
UV 210 nm
UV 254 nm

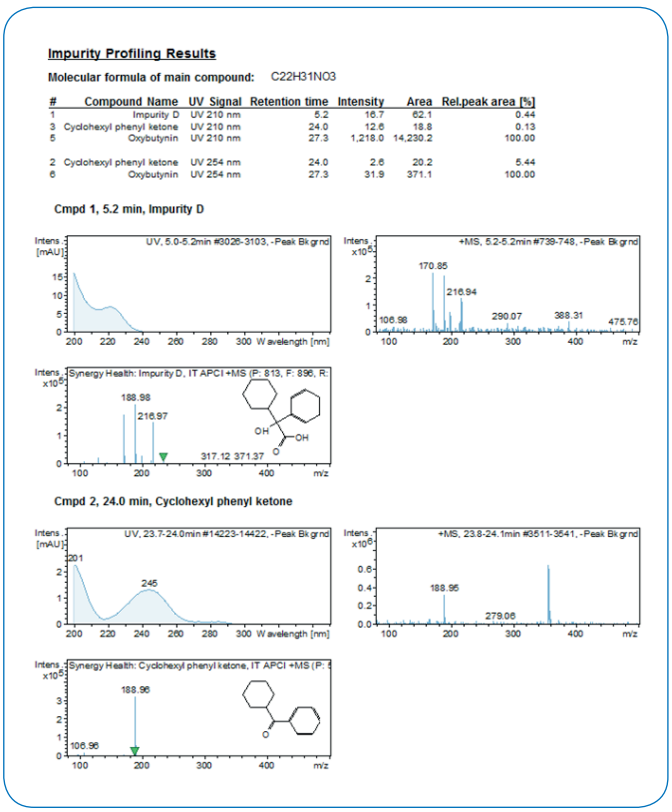
Compound Verification Results

Expected formula of main compound: C22H31NO3
Expected molecular weight of main compound: 357.23 Da

Expected compound detected!

#	Compound Name	Lib. MW	RT [min]	Fit/Purity'	Chromatogram
1	Impurity D	234.13	5.2	896	UV 210 nm
2	Cyclohexyl phenyl ketone	188.12	24.0	824	UV 254 nm
3	Cyclohexyl phenyl ketone	188.12	24.0	670	UV 210 nm
4	Oxybutynin	357.23	27.3	998	AutoMSn(358.27), Masses excluded
5	Oxybutynin	357.23	27.3	879	UV 210 nm
6	Oxybutynin	357.23	27.3	878	UV 254 nm

Fig. 10: First page of the QC report as generated by the Compass OpenAccess QC for API verification.



Impurity Profiling Results

Molecular formula of main compound: C22H31NO3

#	Compound Name	UV Signal	Retention time	Intensity	Area	Rel.peak area [%]
1	Impurity D	UV 210 nm	5.2	16.7	62.1	0.44
3	Cyclohexyl phenyl ketone	UV 210 nm	24.0	12.6	18.8	0.13
5	Oxybutynin	UV 210 nm	27.3	1,218.0	14,230.2	100.00
2	Cyclohexyl phenyl ketone	UV 254 nm	24.0	2.6	20.2	5.44
6	Oxybutynin	UV 254 nm	27.3	31.9	371.1	100.00

Cmpd 1, 5.2 min, Impurity D

UV, 5.0-5.2min #3026-3103, -Peak Bkgrnd
+MS, 5.2-5.2min #739-748, -Peak Bkgrnd
Synergy Health: Impurity D, IT APCI +MS (P: 813, F: 896, R:

Cmpd 2, 24.0 min, Cyclohexyl phenyl ketone

UV, 23.7-24.0min #14223-14422, -Peak Bkgrnd
+MS, 23.8-24.1min #3511-3541, -Peak Bkgrnd
Synergy Health: Cyclohexyl phenyl ketone, IT APCI +MS (P:

Fig. 11: Second part of the QC report providing impurity profiling results: Relative UV peak areas as well as UV and MS spectra of the irradiation products (API spectra not shown here.)

Table 2. Overview of the results from the automated QC analysis of four different oxybutynin samples, showing detected and verified compounds, retention time and relative abundance at 210 and 254 nm, respectively

Sample	Compound	Retention time t_R [min]	Rel. peak area at 210 nm [%]	Rel. peak area at 254 nm [%]
Oxybutynin (control)	oxybutynin	27.4	100	100
Oxybutynin irradiated	oxybutynin	26.7	100	100
	CPK	23.9	0.13	5.44
	impurity D	5.1	0.44	<0.1
Oxybutynin + 0.1% CPK	oxybutynin	27.4	100	100
	CPK	24.0	0.11	5.40
Oxybutynin + 1.0 % CPK	oxybutynin	27.4	100	100
	CPK	24.0	1.24	32.89

These findings indicate that drug purity specifications for unknown impurities solely based on LC-UV analysis may not consider real relative abundances. Implementation of mass spectrometry in the QC workflow allows identification and thus more reliable quantification.
This illustrates the additional value of using more advanced analytical techniques in drug substance QC. As a consequence, new protocols and specifications in accordance with the new technology would be required.

Conclusions

The described LC-UV-Ion trap MS^n approach enables more comprehensive quality control of active pharmaceutical ingredients (API) and other drug substances than LC-UV alone.

Ion trap mass spectrometry allows for the characterization of unknown degradation products based on MS and MS^n data, as shown for cyclohexyl phenyl ketone. MetaboliteTools facilitates the detection of unknown low abundant impurities by detailed and sensitive comparison of treated and non-treated samples. The FragmentationExplorer software supports MS^n spectrum interpretation and thus structure verification of degradation products.

The Bruker Compass OpenAccess software provides a multi-user environment that enables operators new to LC-MS to produce valuable data. Pre-defined workflows reduce the effort for routine QC method development, and the simplified user interface allows performing LC-MS analysis with minimal training. For the quality control of oxybutynin samples a fully automated workflow using UV and MS^n information was established based on the Compass OpenAccess QC software.

Drug compound verification is achieved using the molecular mass as well as MS^n spectral library matching. For impurity profiling the relative peak area of the degradation products is determined from the UV chromatograms at 210 and 254 nm. Confirmation of the identity of the impurities is achieved comparing UV and MS data to a spectral library. This QC spectral library can be easily extended by the user.

As several elements of the described set-up and workflow are generic, this approach offers great potential for routine QC of drug substances other than oxybutynin.

Acknowledgments

We gratefully acknowledge John Hopman for the sample irradiations at the facility of STERIS Applied Sterilization Technologies in Ede, The Netherlands.

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[5] Bruker Daltonics Application Note # ET-31, Drug Metabolite Detection and Identification Combining Bruker MetaboliteTools with Lhasa Limited's Meteor™ Software

[6] Bruker Daltonics Application Note # LCMS-62, Walk-Up Ion Trap Mass Spectrometer System in a Multi-User Environment Using Compass OpenAccess Software

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