(KBr): 3347, 3121, 2953, 1743 br, 1676, 1649, 1557, 1532 cm^{-1}. MS (120 °C): m/z (%) = 354 (0,8; M^+), 312 (3), 253 (2), 212 (1), 196 (4), 180 (2), 179 (2), 166 (2), 162 (2), 159 (9), 154 (5), 140 (2), 136 (4), 124 (5), 122 (3), 110 (3), 109 (2), 101 (21), 86 (100), 72 (5), 70 (5), 58 (38), 44 (75). ^{1}H-NMR (DMSO-d_6): δ (ppm) = 7,74 (s, 1 H, 2′-H); 7,59 (q, 1 H, H_3CNHCO, ^3J = 4,5, aust.); 5,4–5,2 (m, 1 H, 2-H_M); 4,61 (dd, 1 H, 1-$H_{A'}$, $^2J_{A'/B'}$ = 12,0, $^3J_{A'/M}$ = 3,7); 4,35 (dd, 1 H, 1-$H_{B'}$, $^3J_{B'/M}$ = 6,0); 4,19 (dd, 1 H, 3-H_A, $^2J_{A/B}$ = 14,2, $^3J_{A/M}$ = 2,9); 4,06 (dd, 1 H, 3-H_B, $^3J_{B/M}$ = 8,4); 3,01 (s, 3 H, $H_3CNCOCH_3$); 2,76 (d, 3 H, H_3CNHCO, nach D_2O-Aust. s); 2,04 (s, 3 H, $OCOCH_3$); 1,93 (s, 3 H, $OCOCH_3$); 1,75 (s, 3 H, $H_3CNCOCH_3$).
$C_{15}H_{22}N_4O_6$ (354,4)

3.25. *1-Methyl-4-methylamino-imidazol-5-carbonsäure-Natriumsalz* (36)

4,8 g (28,5 mmol) **6** werden in 50 ml 30 proz. NaOH 9 h auf 100 °C erhitzt. Nach dem Abkühlen wird der Niederschlag abgetrennt, vorsichtig mit Eiswasser gewaschen und getrocknet. Schmp. > 300 °C. Rohausbeute: 3,5 g (69%). IR (KBr): 3389, 3112, 2952, 2817, 1636, 1588, 1528, 1512 cm^{-1}. MS (230 °C): m/z (%) = 111 (82), 110 (66), 96 (5), 95 (5), 83 (39), 82 (32), 69 (32), 56 (10), 45 (77), 43 (100).

3.26. *1,2,4,7-Tetramethyl-imidazo[1,5-a]pyrimidinium-diperchlorat* (38 · 2 ClO_4)

Nach AAV 1c: 0,2 g (1,13 mmol) **36**, 0,23 g (2,3 mmol) **4**, 3 ml (3 mmol) 1 N $HClO_4$/Eisessig. Beim Ansäuern entweicht ein Gas. Gelbe Nadeln aus CH_3CN/Ether. Schmp. 237 °C (Zers.). Ausb. 0,2 g (47%). IR (KBr): 3416, 3126, 3081, 3011, 2926, 1663, 1574, 1522 cm^{-1}. MS (Substanz nicht verdampfbar, Zersetzung im Tiegel): m/z (%) = 67 (6), 50 (55), 44 (100). ^{1}H-NMR (CD_3CN): δ (ppm) = 9,52 (s, 1 H, 6-H); 8,32 (′d′, 1 H, 8-H, ′J′ = 1,6); 7,49 (s, 1 H, 3-H); 4,27 (s, 3 H, 7-CH_3); 4,17 (s, 3 H, 1-CH_3); 2,93 (s, 3 H, 2-CH_3*); 2,90 (s, 3 H, 4-CH_3*). UV (CH_3CN): λ_{max} = 235 (4,53), 242 sh (4,50), 329 (3,48).
$C_{10}H_{15}N_3^{2+} \cdot 2\ ClO_4^-$ (376,2)

Herrn Dr. P. Schmitt, Bayer AG Wuppertal, danken wir für die Aufnahme von korrelierten NMR-Spektren und dem Fonds der Chemischen Industrie für die Unterstützung dieser Arbeit.

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Structure elucidation of "related substances" of acebutolol by LC-MS/MS

C. Endres[1], M. Herderich[2] and S. Ebel[1]

A liquid chromatographic method for the separation of "related substances" of acebutolol hydrochloride is described. Tandem mass spectrometry in combination with liquid chromatographic separation proved to be a powerful method for the elucidation of chemical structures of the impurities.

1. Introduction

In Pharmacopoeias, one of the more important criteria to assess the purity, and thus the quality of a chemical substance is the test for so-called "related substances". "Related substances" are intermediates and by-products from a synthetically produced organic substance, coextracted substances from a natural product and degradation products of the substance [1]. Useful limitation of the levels of individual impurities and a sum of impurities requires knowledge of their pharmacological and perhaps toxic effects. The identification of all recurring impurities at or above the 0.1 percent level – toxic impurities even below this level – is according to the ICH guideline "Impurities in New Drug Substances" [2] an objective of the "Technical Guide for the Elaboration of Monographs, 2nd Edition" [1]. For such structural characterisation, mass spectrometry, a method which is already included in the USP 23 and is likely to be attended to the European Pharmacopoeia, particularly in combination with gas- or liquid chromatography is a powerful method for structural characterisation.

Acebutolol (AC), (*RS*)-3′-Acetyl-4′-[2-hydroxy-3-(isopropylamino)propoxy]butyranilide (**1**), is a cardio-selective β-adrenergic blocking drug with intrinsic sympathomimetic activity and membrane-stabilizing properties [3–5]. The daily oral doses are ranging from 200 mg up to 1200 mg [6, 7]. Thus, even a small percentage of impurities are adsorbed in considerable amounts.

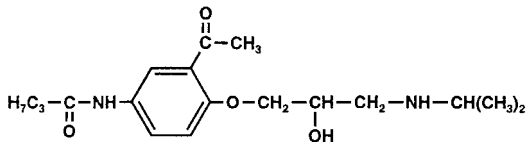
In the monograph AC of the European Pharmacopoeia [8] substances **2**–**6** are described as possible impurities.

The intention of this paper is to describe the structural assignment of possible impurities and characterisation of unknown "related substances" of examined batches of the drug AC by triple-quadrupole mass spectrometry after separation by reversed phase liquid chromatography.

2. Investigations, results and discussion

2.1. *Chromatographic performance*

The first objective was to develop a fast, cheap and reproducible chromatographic method that is proved to be suited for the separation of all included impurities from AC and to facilitate coupling with a mass spectrometer. Like most of the other β-adrenergic blocking drugs AC is administered as a salt of an organic base. Therefore a chroma-



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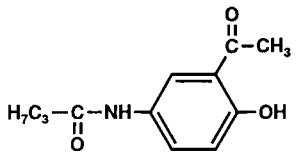
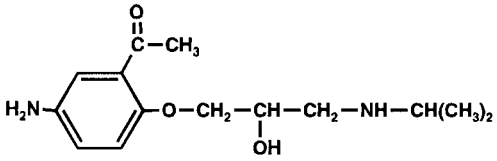
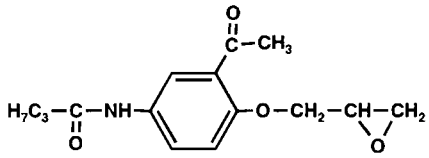


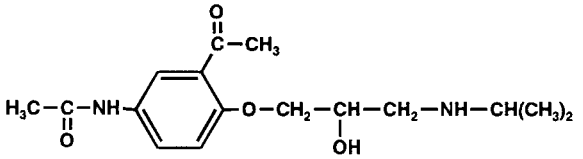
3´-acetyl-4´-hydroxybutyranilide (**2**)



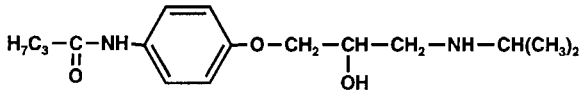
5´-amino-2´-[2-hydroxy-3-(1-methylethylamino)-propoxy]acetophenone (Acetolol) (**3**)[10]



3´-acetyl-4´-(2,3-epoxypropoxy)-butyranilide (**4**)



3´-acetyl-4´-[2-hydroxy-3-(1-methylethylamino)propoxy]-acetanilide (Diacetolol) (**5**)



4´-[2-hydroxy-3-(1-methylethylamino)propoxy]butyranilide (**6**)

tographic system was developed, using a reversed phase column and aqueous ammonium acetate as buffer. Aqueous ammonium acetate is a suitable buffer for reversed phase chromatography. It is cheap, soluble in acetonitrile and volatile, which facilitates coupling of the LC-separation to mass spectrometry. An endcapped octadecyl silicagel was chosen as column packing material to avoid increased chromatographic peak tailing. The pH-value was adjusted to 4.5 with acetic acid for optimisation of the impurities' separation. Detection wavelength was 236 nm. Two typical chromatograms are shown in Figs. 1 and 2.
Relative retention times of the impurities detected by the UV method in four different batches of AC are summarized in Table 1; subsequently MS and MS/MS analyses were performed to achieve the structural assignment of these impurities.

2.2. Mass spectrometric investigation

Molecules were evaporated from the mobile phase and ionized by electrospray ionisation (ESI). The protonated molecules $[M+H]^+$ were transferred to a triple-quadrupole tandem mass spectrometer and analysed (Table 2). Due to their low abundances, *impurities d* and *i* were not characterized further.
The molecular mass of the "related substances" can directly be deduced from the protonated molecules $[M+H]^+$. As starting point for more detailed structural characterisation we used the assumption, that the chemical structure of any "related substance" is related to the pure substance [9]. For MS/MS analysis protonated molecules $[M+H]^+$ are selected in quadrupole 1, collision activated decomposition with argon as inert collision gas in quadrupole 2 leads to characteristic product ions, which are scanned in quadrupole 3.
Fig. 3 shows the resulting fragment ions of AC, which are clearly resembling characteristic chemical substructures of the analyte. This knowledge proved to be essential for structural assignment of unknown "related substances", because matching or related fragment ions can be detected in the respective product ion spectra [9].
In case of AC, product ion m/z 319 is obtained by the elimination of H_2O. Elimination of 1-methylethylamine (m/z 278) and H_2O yield the product ion m/z 260. Further cleavage of the aromatic acetyl group results in the product ion m/z 218. The abundant product ion m/z 116 is representing the 2-hydroxy-3-(1-methylethylamino)propyl-side chain [10], which is additionally characterised by the product ions m/z 98 (m/z 116-H_2O) and m/z 72 $[CH_2{=}NH{-}CH(CH_3)_2]^+$ [15].
Impurity b forms a protonated molecule $[M+H]^+$ m/z 267. The presence of product ions m/z 116, m/z 98, and m/z 72 indicates the intact 2-hydroxy-3-(1-methylethylamino)propyl-side chain (Fig. 4). The chromatogram obtained after acidic hydrolysis of the AC-solution supports this structure assignment (Fig. 5).
MS/MS analysis of *impurity c* again reveals the characteristic product ions m/z 116, m/z 98, and m/z 72 which are indicative for the complete side chain (Fig. 6) of the protonated molecule m/z 309 $[M+H]^+$. Replacing the butyric- by an acetyl-group is expected to count responsible for product ions m/z 232 and m/z 190 instead of m/z 260 and m/z 218, the latter being formed after collision activated decomposition of the protonated molecule of AC (m/z 337). Consequently, the product obtained by acidic hydrolysis and subsequent acetylation of AC supports the identification of impurity c as demonstrated by the chromatographic analysis in Fig. 7.
Impurities e and *g* are both characterized by the protonated molecule m/z 295 $[M+H]^+$. Regarding the identification of these "related substances" the respective product ion spectra provide more detailed information (Figs. 8 and 9).
The product ion spectrum of *impurity e* indicates a change in the 2-hydroxy-3-(1-methylethylamino)propyl-side chain as indicated by the missing product ions m/z 116, m/z 98, and m/z 72. However, fragments m/z 74 and m/z 56 demonstrate the presence of an aminohydroxypropyl moiety. In addition, loss of the unsubstituted amino function (m/z 278) and subsequent loss of H_2O (m/z 260) support the structural assignment of "related substance" e (4′-(3-amino-2-hydroxypropoxy)butyranilide). Presence of impurity e can be explained by a reaction with ammonium hydroxide during synthesis of AC [3, 11–14] – probably caused by contaminated reagents.

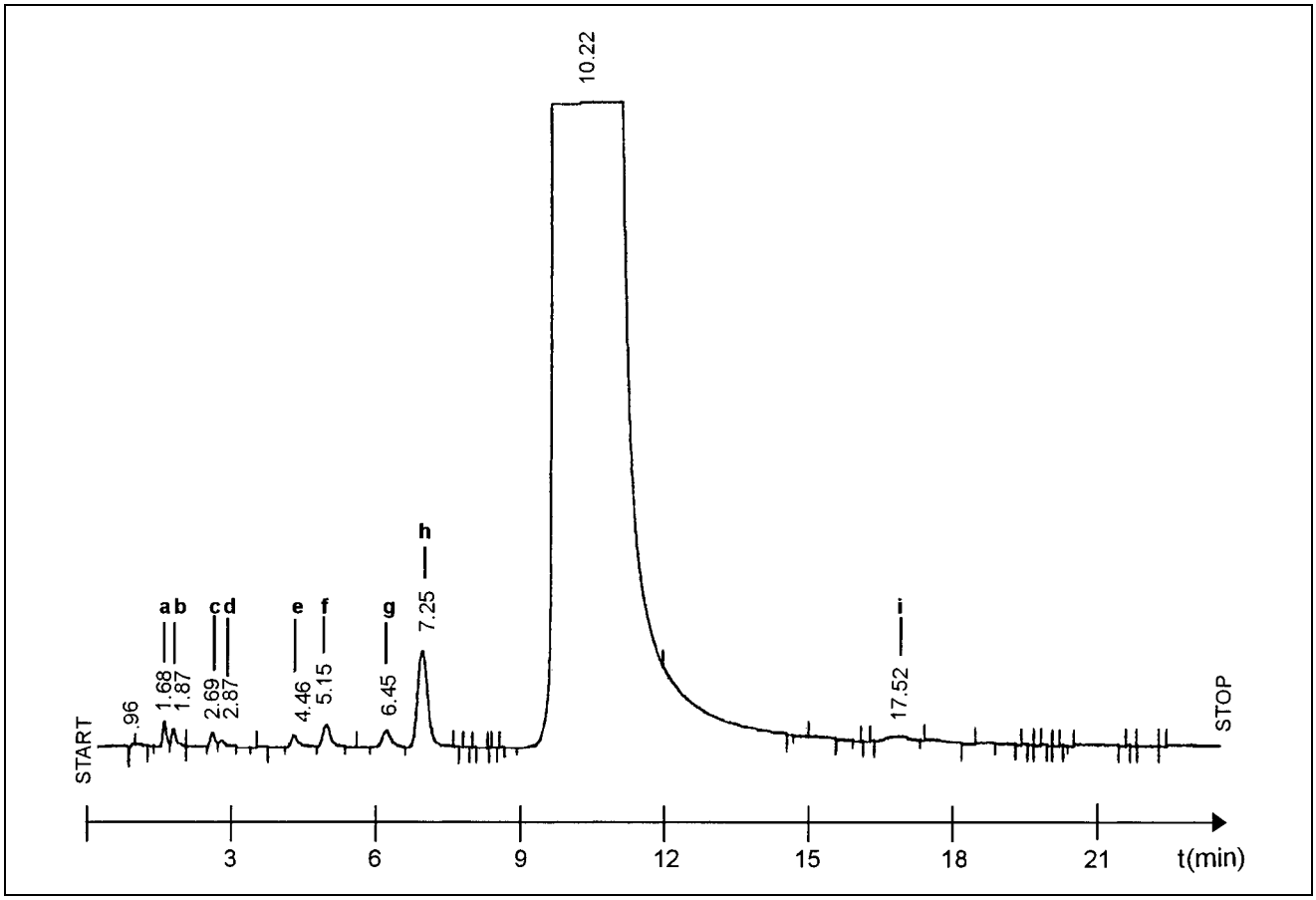


Fig. 1: HPLC-UV-chromatogram of AC (batch A)

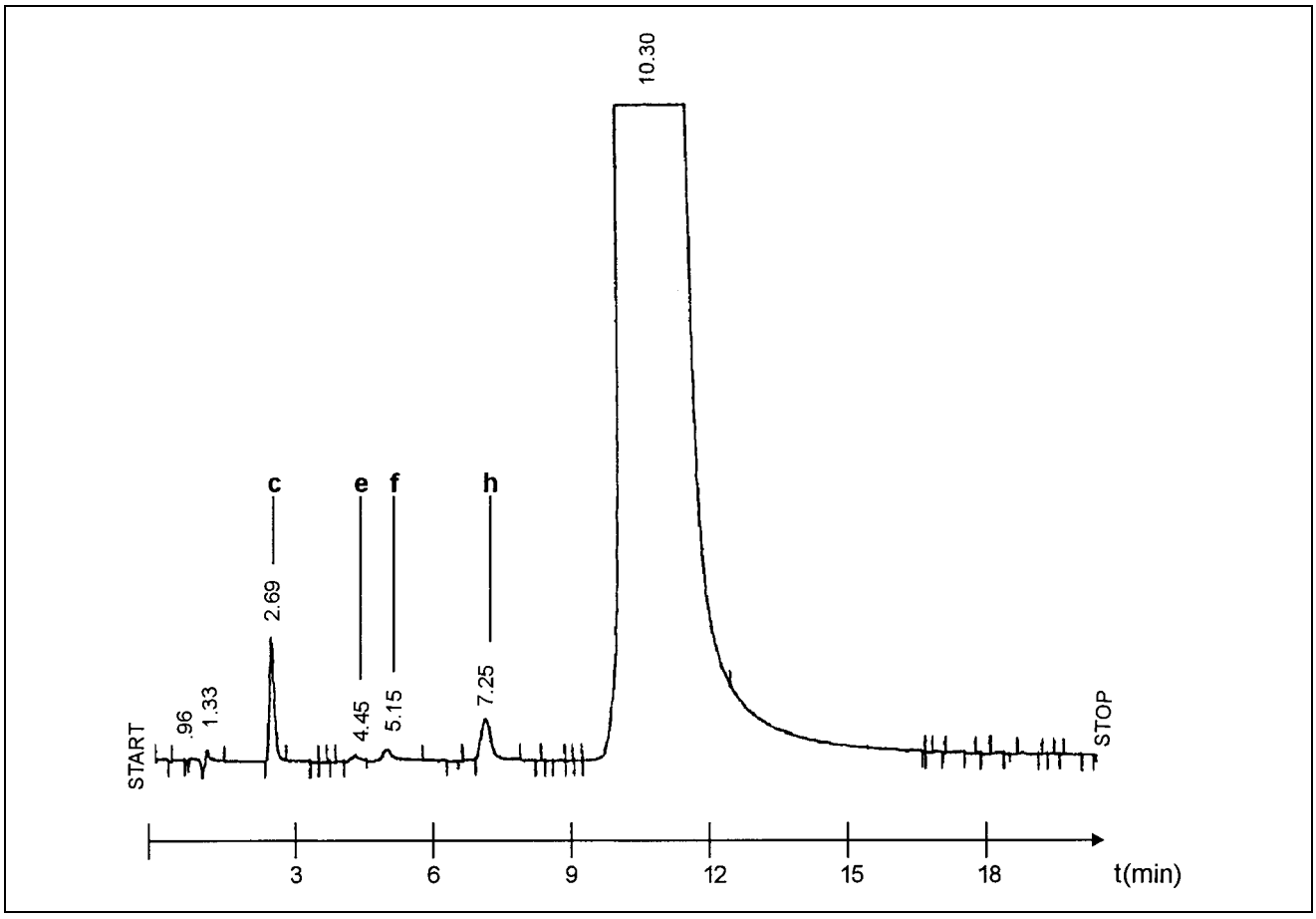


Fig. 2: HPLC-UV-chromatogram of AC (batch D)

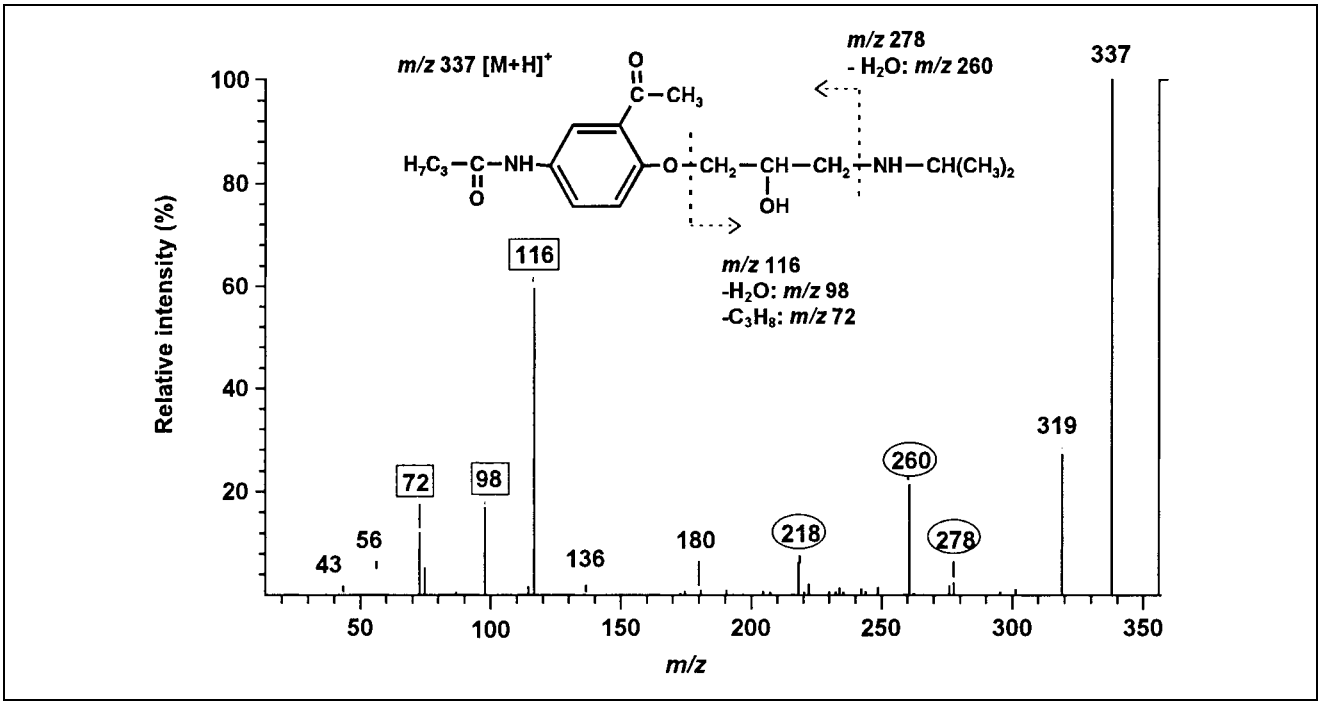


Fig. 3: Product ion spectrum of AC (precursor ion m/z 337 $[M+H]^+$)

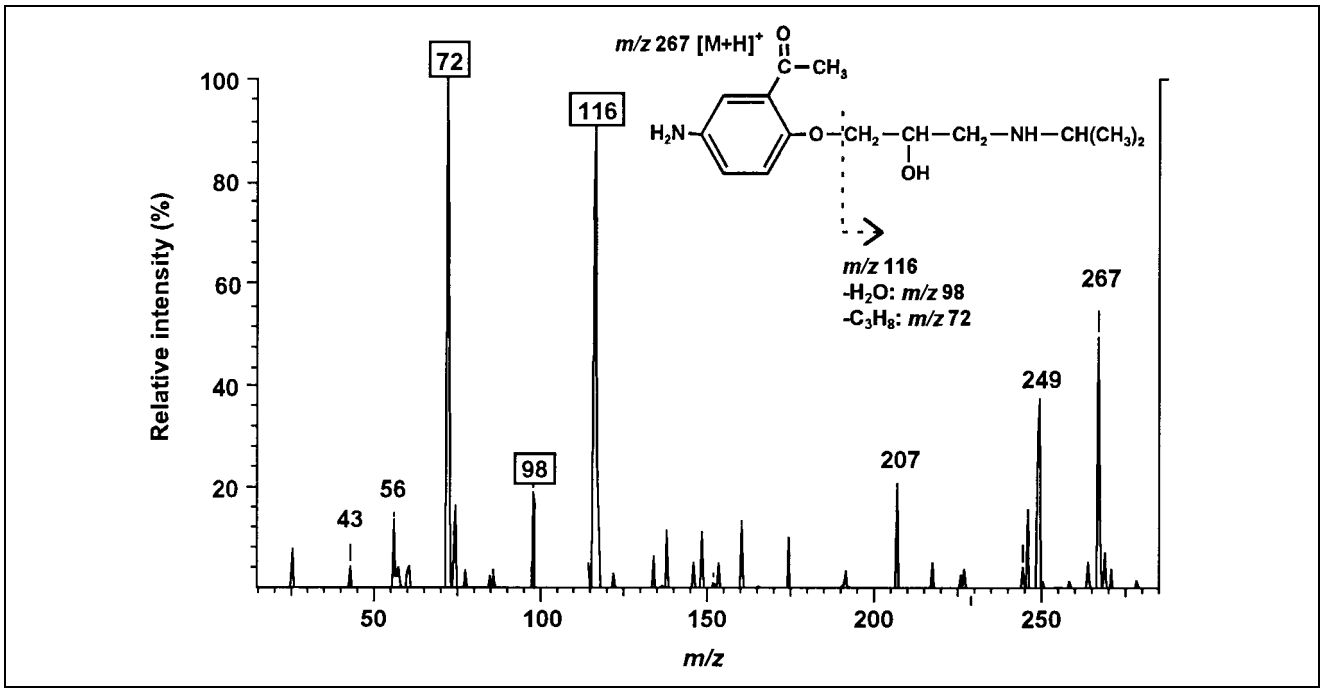


Fig. 4: Product ion spectrum of *impurity b* (precursor ion m/z 267 $[M+H]^+$)

Table 1: Relative retention times of the impurities of acebutolol hydrochloride

Batch	0.16	0.18	0.26	0.28	0.43	0.5	0.63	0.71	1	1.71
A	a	b	c	d	e	f	g	h	AC	i
B	a	b	c		e	f	g	h	AC	
C					e	f		h	AC	
D			c		e	f		h	AC	

Table 2: Protonated molecules $[M+H]^+$ performed by ESI

a	b	c	d	e	f	g	h	AC	i
341	267	309		295	323	295	323	337	

Typical for the product ion spectrum of *impurity g* (Fig. 9) are product ions m/z 116, m/z 98, m/z 72 and m/z 253. Instead of product ion m/z 260 product ion m/z 218 can be observed while no further loss of ketene (m/z 218–42; corresponding to m/z 260–42 in Fig. 3) is detectable. Likely, the difference

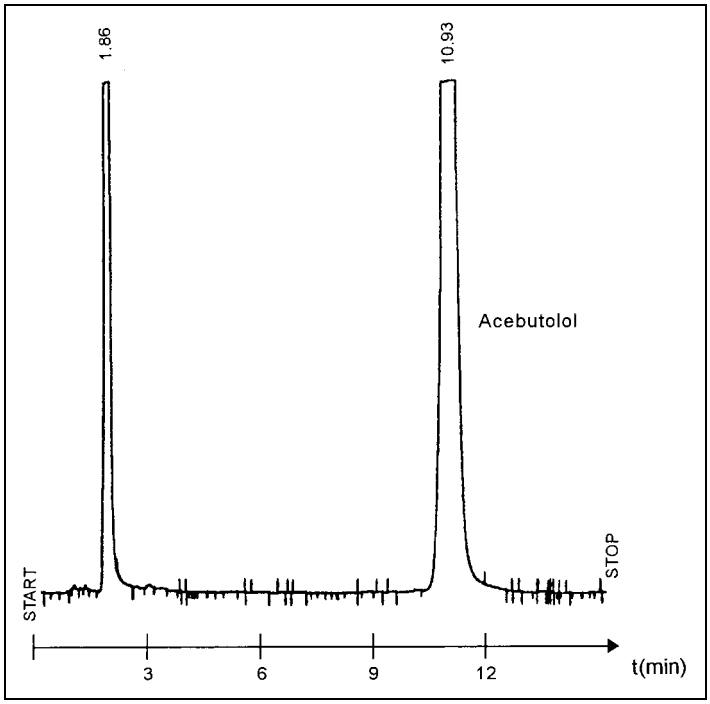


Fig. 5:
HPLC-UV-chromatogram of the hydrolysed test solution

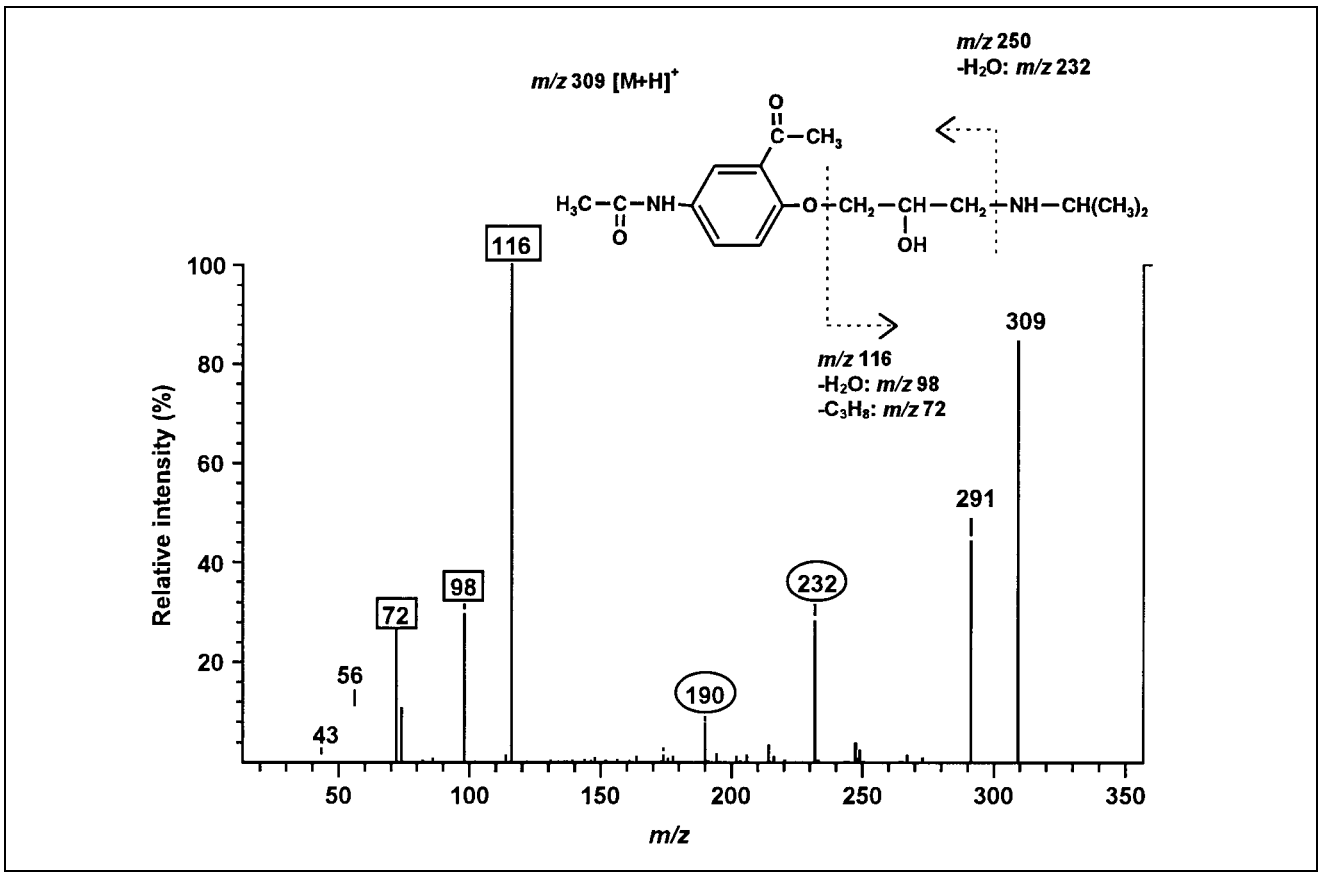


Fig. 6: Product ion spectrum of *impurity c* (precursor ion m/z 309 $[M+H]^+$)

is the result of the missing aromatic acetyl group. Thus, in accordance to the impurity listed in the European Pharmacopoeia, "related substance" g can be 4′-[2-hydroxy-3-(1-methylethylamino)propoxy]-butyranilide (**6**).

Both *impurities f* and *h* yield protonated molecules $[M+H]^+$ m/z 323. Concerning impurity f, m/z 260 and m/z 218 are missing, but the corresponding product ions m/z 246 and m/z 204 are apparent along with m/z 116, m/z 98, and m/z 72. Hence, the compound is likely to be modified in the butyranilide moiety. According to the synthesis [3, 11–14] impurity f could be characterised as 3′-acetyl-4′-[2-hydroxy-3-(1-methylethylamino)propoxyl]-2,3-epoxypropylanilide (**7**), alternatively the butyranilide moiety could be replaced by n-butyl- or propionylanilide.

In the product ion spectrum of impurity h ions m/z 116, 98, and 72 are replaced by m/z 102, m/z 84, and m/z 58, but m/z 260 and m/z 218 are present. Here the structural differences should affect the 2-hydroxy-3-(1-methylethyl-

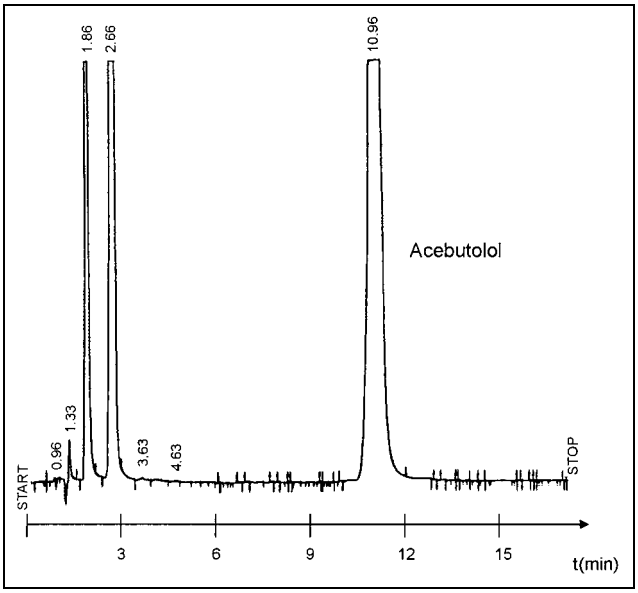
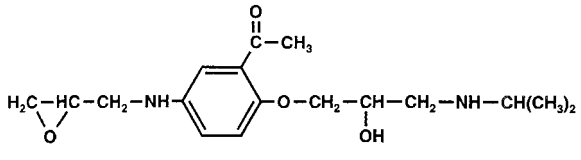
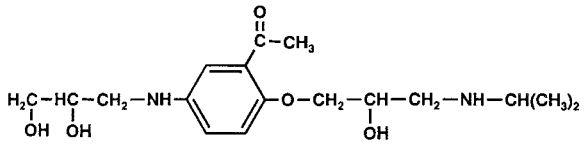


Fig. 7: HPLC-UV-chromatogram of the hydrolysed and subsequently acetylated AC

amino)propyl-side chain. Obviously, ethylamine – the corresponding "related substance" for the β-adrenergic blocking drug metroprolol tartrate is listed in the European Pharmacopoeia [8] – replaces isopropylamine.
Conclusive identification of *impurity a* ($[M+H]^+$ m/z 341) is difficult, but product ions m/z 116, m/z 98, m/z 72, and m/z 264 and m/z 222 (Fig. 12) indicate structural similarity to impurity f. Correspondingly, we propose that impurity a could represent a triol **8** as depicted below.
In addition, the relative retention times (Table 3) support the proposed structures for the respective "related substances".



(7)

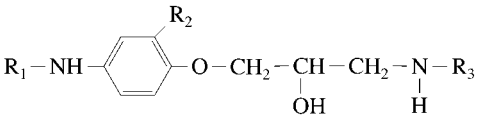


(8)

2.3 Conclusion

With the presented chromatographic method all impurities are clearly separated from AC. Application of a volatile buffer enabled the detailed mass spectrometric characterisation of the "related substances" detected by UV. Product ions as obtained by collision activation of AC allowed reliable identification of structural motifs and proved to be beneficial for further characterisation of impurities b, c, e,

Table 3: Correlation between the relative retention times and the proposed structural characterisations of the "related substances"



	$[M+H^+]$	t_R	R_1	R_2	R_3
Impurity a	341	0.16	$H_2C-HC-H_2C-$ (OH, OH)	$-C(=O)-CH_3$	$-CH(CH_3)_2$
Impurity b	267	0.18	$H-$	$-C(=O)-CH_3$	$-CH(CH_3)_2$
Impurity c	309	0.26	$H_3C-C(=O)-$	$-C(=O)-CH_3$	$-CH(CH_3)_2$
Impurity e	295	0.43	$H_7C_3-C(=O)-$	$-C(=O)-CH_3$	$-H$
Impurity f	323	0.5	$H_2C-HC-H_2C-$ (O)	$-C(=O)-CH_3$	$-CH(CH_3)_2$
Impurity g	295	0.63	$H_7C_3-C(=O)-$	$-H$	$-CH(CH_3)_2$
Impurity h	323	0.71	$H_7C_3-C(=O)-$	$-C(=O)-CH_3$	$-C_2H_5$
AC	337	1	$H_7C_3-C(=O)-$	$-C(=O)-CH_3$	$-CH(CH_3)_2$

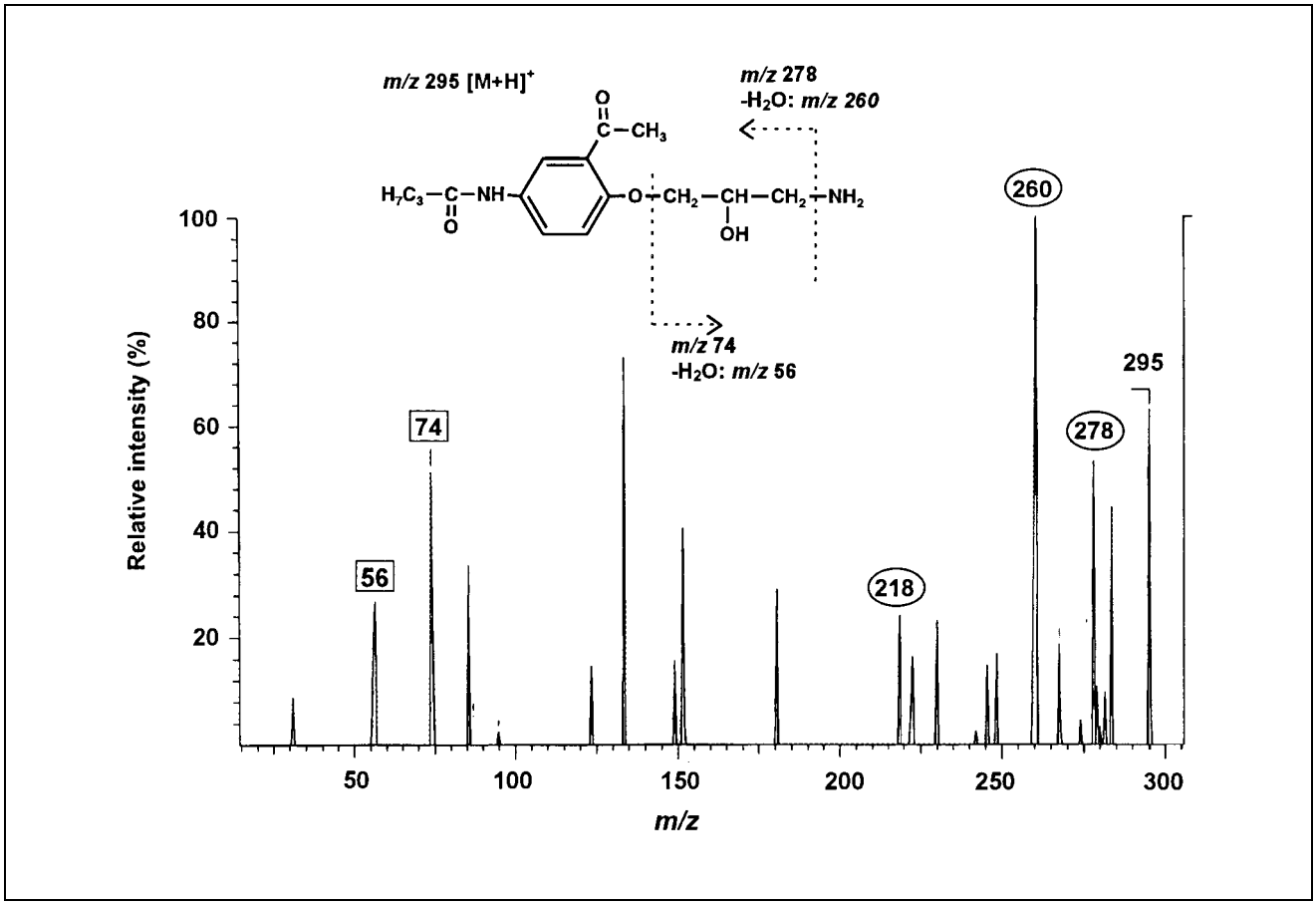


Fig. 8: Product ion spectrum of *impurity e* (precursor ion m/z 295 $[M+H]^+$)

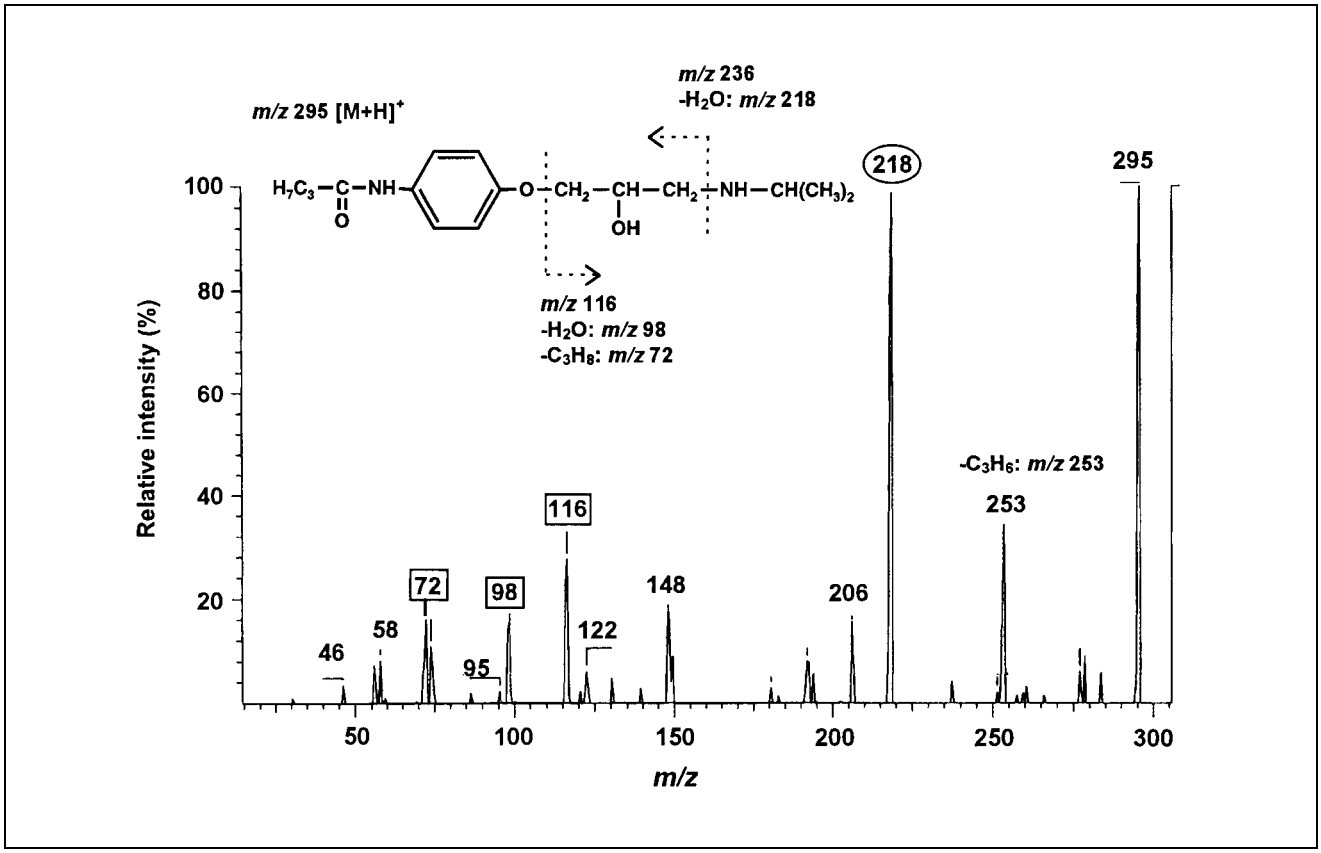


Fig. 9: Product ion spectrum of *impurity g* (precursor ion m/z 295 $[M+H]^+$)

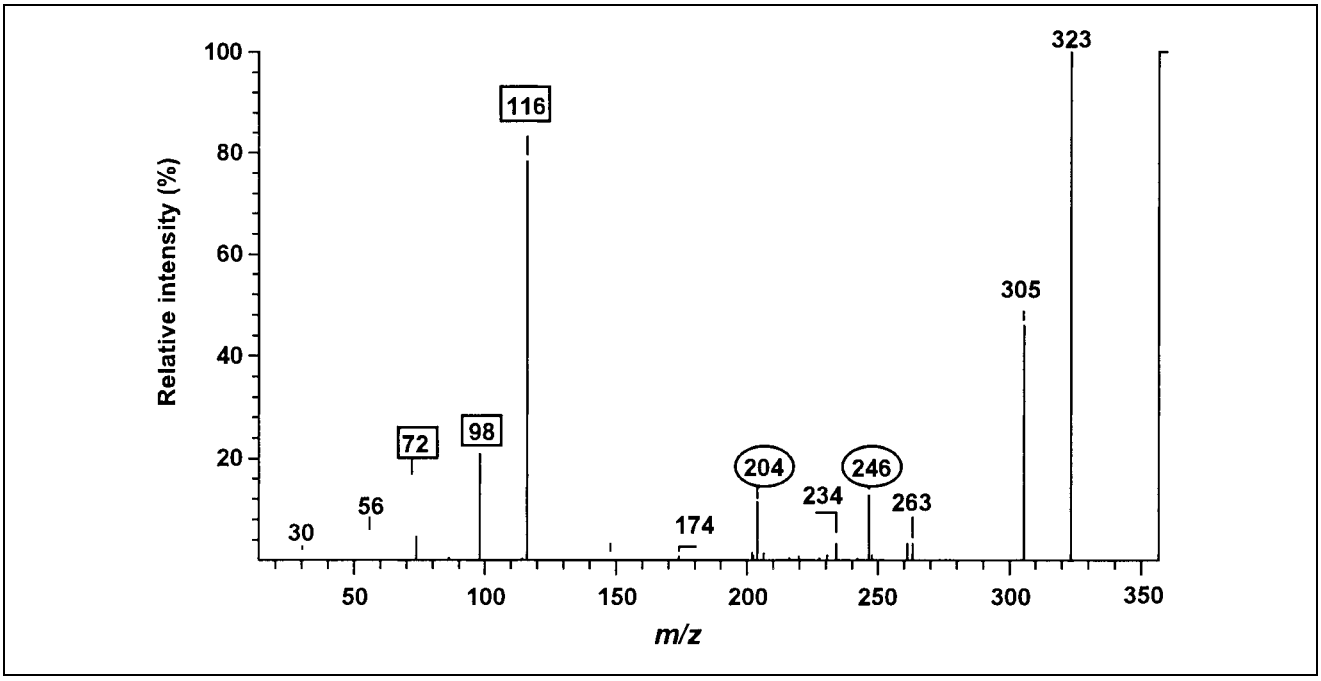


Fig. 10: Product ion spectrum of *impurity f* (precursor ion m/z 323 $[M+H]^+$)

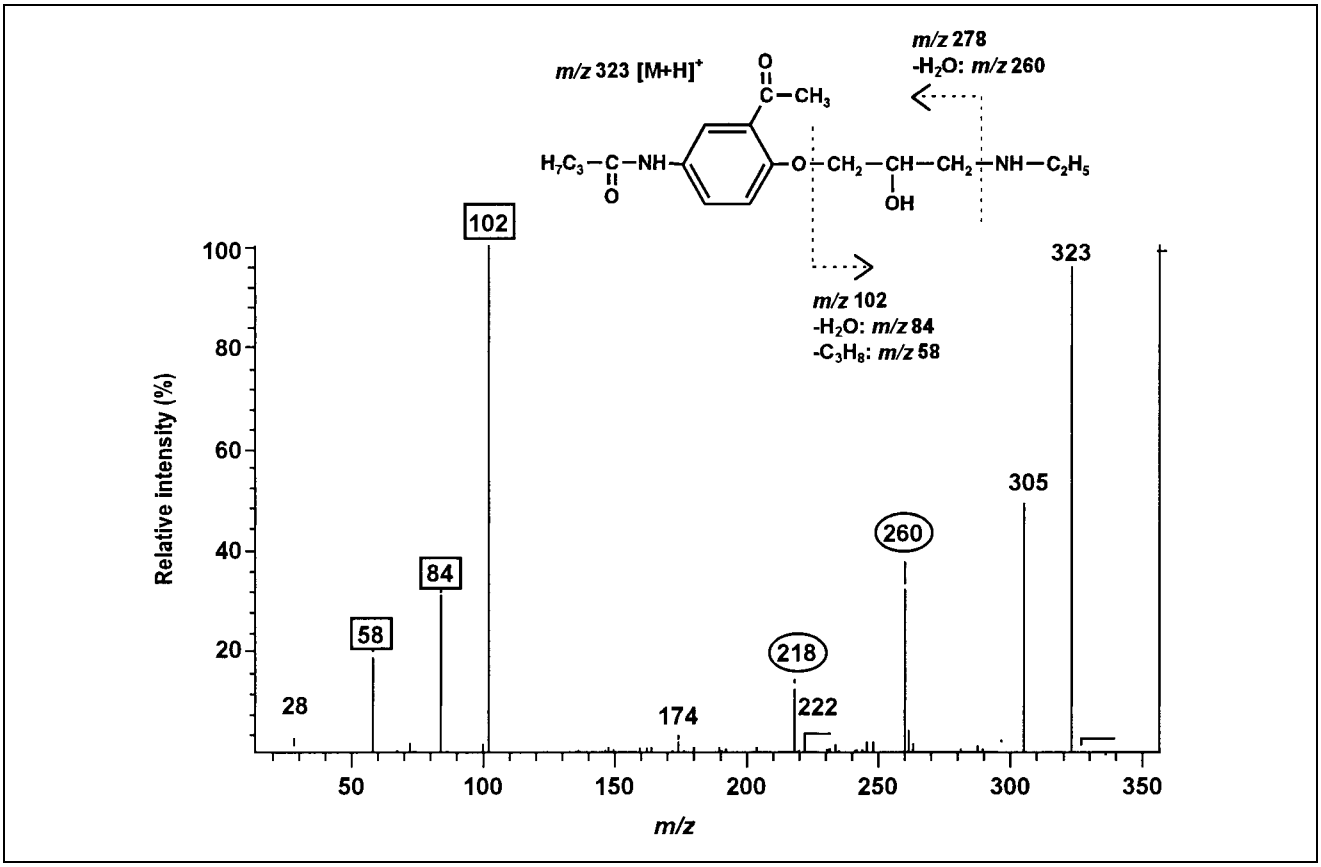


Fig. 11: Product ion spectrum of *impurity h* (precursor ion m/z 323 $[M+H]^+$)

g, and h. In addition, identity of impurities b and c (acetolol and diacetolol) was confirmed by model reactions. On the basis of the product ion spectra structures were proposed for impurities a and f. However, conclusive identification of these "related substances" requires additional information concerning the respective synthesis of by-products.

3. Experimental

3.1. Sample preparation

The test solution for the development of the purity test was prepared by dissolving 10.0 mg AC in 10.0 ml mobile phase. Degradation by hydrolysis was performed as follows: The test solution (1.0 ml) was carefully heated with 5 ml of 0.1 M HCl to dryness and finally dissolved again in 20 ml mobile phase. The subsequent acetylation was done by adding

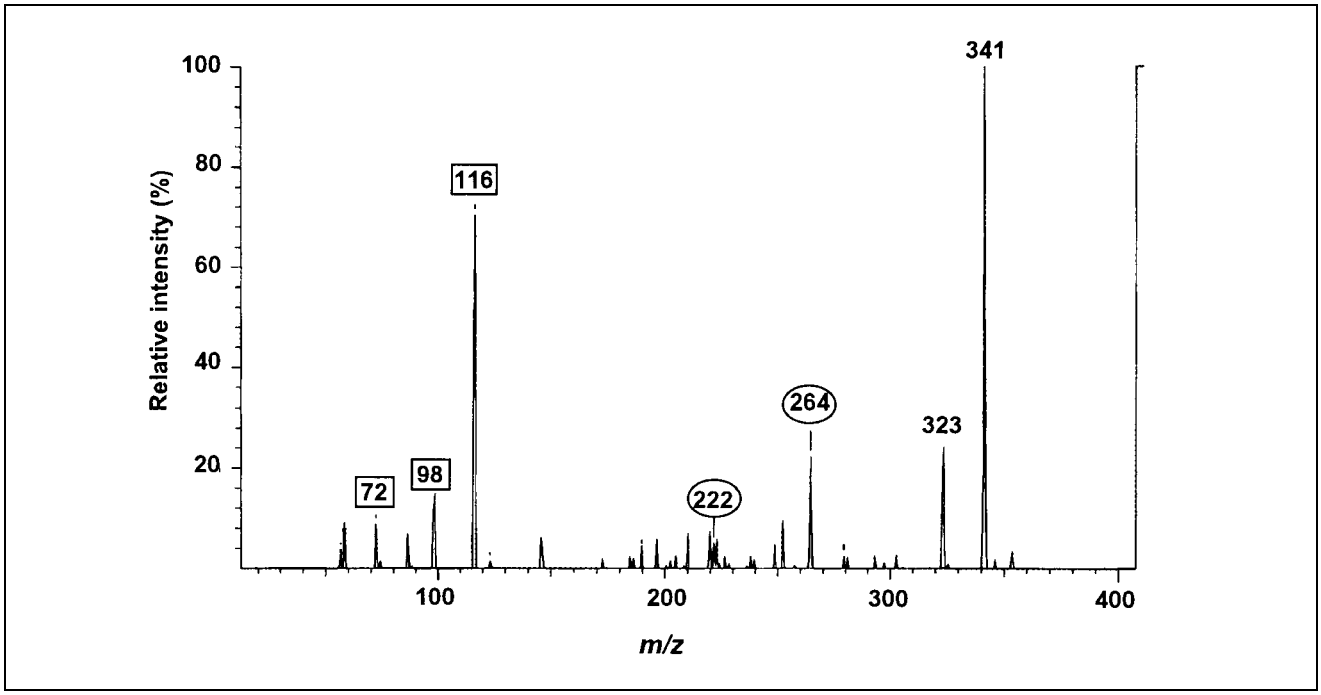


Fig. 12: Product ion spectrum of *impurity a* (precursor ion m/z 341 $[M+H]^+$)

0.2 ml of a 0.1% (v/v) solution of acetic anhydride in mobile phase. For the mass spectroscopic examination 20 mg of AC were dissolved in 10 ml mobile phase.

3.2. Chromatographic conditions

Separations were performed on a 5 µm LiChrospher 100 RP 18 e column (125 × 4.0 mm i.d.; Merck, Darmstadt, Germany). The mobile phase was a mixture of 86 volumes of an aqueous solution of ammonium acetate (7700 mg/l) adjusted to pH 4.5 with acetic acid and 14 volumes of acetonitrile, at a flow rate of 1.0 ml/min. The detection wavelength was 236 nm and all analyses were carried out isocratically.
For LC-MS/MS experiments an aqueous solution of ammonium acetate (390 mg/l) adjusted to pH 4.5 with acetic acid was used.

3.3. Apparatus

The HPLC apparatus consisted of an Abimed Gilson Pump 307, an injection valve equipped with a 20 µl sample loop (Rheodyne, manual switching, Bischoff, Leonberg), and for UV-detection a Knauer Variable Wavelength Monitor, model 73287 (Knauer, Berlin). Integration was performed by an HP 3394 integrator (Hewlett Packard, Böblingen).
A triple quadrupole mass spectrometer equipped with electrospray interface (Finnigan TSQ 7000, Finnigan MAT, Bremen) was used. Nitrogen served both as sheath and aux gas, and argon (2 m Torr) was applied as collision gas.

3.4. Chemicals

The following chemicals were used: Acetic acid 99–100% analyzed HPLC-Reagent (Baker, Griesheim, Germany); acetic anhydride p.a., ACS, Reag. ISO, Pharm. Eur. (Riedel de Haen, Seelze, Germany); acetonitrile Lichrosolv® for chromatography (Merck, Darmstadt, Germany); ammonium acetate puriss. p.a. ACS; ≥98% (Fluka, Deisenhofen, Germany); 0.1 M HCL (Grüssing, Filsum, Germany); Millipore®-water (Millipore GmbH, Eschborn, Germany).

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