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LC–ESI-MS/MS characterization of strophanthin-K

Giorgio Grosa, Gianna Allegrone*, Erika Del Grosso

Dipartimento di Scienze Chimiche, Alimentari, Farmaceutiche e Farmacologiche—Università degli Studi del Piemonte Orientale "Amedeo Avogadro", Via Bovio 6, 28100 Novara, Italy

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Abstract

A liquid chromatography–mass spectrometry (LC–MS) method was developed for the characterization of strophanthin-K, a mixture of cardiac glycosides extracted from the seeds of *Strophanthus kombè*. The method is based on the separation of the cardenolides using high performance liquid chromatography (HPLC) followed by detection with electrospray ionization mass-spectrometry (ESI-MS). Chromatographic separation of the analytes was achieved on a RP C-18 column using water: 1% formic acid in water (v/v):acetonitrile gradient mobile phase. Strophanthin-K glycosides studied in ESI-MS in negative ion mode formed abundant adduct ions $[M + HCOO]^-$ while the pseudomolecular ions $[M - H]^-$ are obtained in ESI-MS/MS experiments. Six different cardiac glycosides were identified and characterized: k-strophanthoside, k-strophanthin-β, helveticoside (erysimin), erysimoside, cymarin and neoglucoerysimoside.

Forced degradation investigations done with strophanthin-K showed that k-strophanthidin (the aglycone of strophanthin-K glycosides) was the main product of degradation in acidic conditions; however, in basic conditions, the hydrolysis of the unsaturated 17β-lactones to the corresponding γ-hydroxy acids was the predominant degradation pathway.

Keywords: Strophanthin-K; k-Strophanthoside; Cardiac glycosides; Cardenolides; LC–ESI-MS/MS

1. Introduction

The cardiac glycosides are a class of naturally occurring drugs whose actions include both beneficial and toxic effects on the heart. Since the desirable cardiotonic action is of particular benefit in the treatment of congestive heart failure and associated oedema, cardiac glycosides represent one of the most important drug classes available.

Among these ones, the mixture of glycosides extracted from the seeds of *Strophantus kombé*, a climbing African plant belonging to the Apocynaceae family, is called strophanthin-K [1].

The therapeutic effects of all cardiac glycosides on the heart are qualitatively similar, but differ strongly in their pharmacokinetic properties, which mainly reflect their polarity. In particular, strophanthin-K is characterized by a short lasting-effect and by a greater diuretic power, which is esteemed of value in cases complicated by oedema [2].

The composition of the mixture was found to vary, k-strophanthoside being the major component. Other glycosides (Fig. 1), containing the same aglycone strophanthidin but with different sugars moiety, were found in strophanthin-K: cymarin, k-strophanthin-β, erysimoside, helveticoside and glucoerysimoside [3]. A little confusion was still present around the structure of this last glycoside as other authors [4] described instead the occurrence of neoglucoerysimoside in *Strophantus kombè* extract.

Cardenolides are also thermally labile and polar compounds: these features prevented their characterization by traditional electron-impact mass spectrometry. The development of more soft-ionization techniques allowed the detection and the characterization of this class of compounds. Indeed digoxin, digitoxin and other related glycosides were studied by FAB-MS [5] and also determined in biological fluids by hyphenated techniques as HPLC–ionspray mass

* Corresponding author. Tel.: +39 0321375855; fax: +39 0321375821.
E-mail address: allegrone@pharm.unipmn.it (G. Allegrone).

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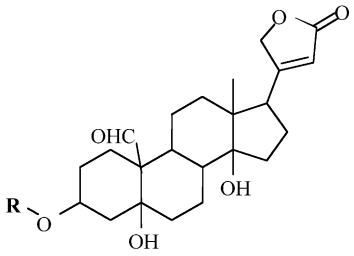

Compound	R	MW	Name	BF
1	Cym-Gluc-Gluc	872	K-Strophanthoside	$C_{42}H_{64}O_{19}$
2	Cym	548	Cymarin	$C_{30}H_{44}O_{9}$
3	Digit	534	Helveticoside	$C_{29}H_{42}O_{9}$
4	H	404	Strophanthidin	$C_{23}H_{32}O_{6}$
5	Digit-Gluc	696	Erysimoside	$C_{35}H_{52}O_{14}$
6	Cym-Gluc	710	K-Strophanthin-β	$C_{36}H_{54}O_{14}$
7	Digit-Gluc-Gluc	858	Neoglucoerysimoside	$C_{41}H_{62}O_{19}$

Fig. 1. Structures of the aglycone and glycosides from *Strophantus kombé* extract (strophanthin-K). Cym: cymarose, Gluc: glucose, Digit: digitoxose.

spectrometry [6] and HPLC–ESI-MS/MS [7]. However, mass spectrometric characterization of strophanthin-K glycosides was not so far reported in literature. Moreover, even if strophanthin-K has been described in a monography of the Italian Pharmacopoeia 9th edition [8], a certified reference standard was not available leading some difficulties in characterizing the composition of the different batches of production of extracts and of the potential degradation products.

In this report, an LC–ESI-MS/MS in negative ion mode method has been developed for the characterization of strophanthin-K glycosides as well as the degradation products of its main components obtained under stressed conditions.

2. Experimental

2.1. Chemicals

Methanol, acetonitrile (all HPLC grade), formic acid (50%, LC–MS grade), k-strophanthoside, helveticoside (erysimin), cymarin, were purchased from Sigma-Aldrich (Milano-Italy); strophanthidin was from ICN (Milano-Italy).

Sample of strophanthin-K was obtained from Pharmafar s.r.l. (Torino-Italy). Water was purified by Milli-Q instrument (Millipore Corp., Bedford, MA, USA).

2.2. Electrospray mass spectrometry (ESI-MS)

The ESI-MS spectra were acquired in negative ion mode using a Thermo Finnigan LCQ Deca XP *plus* Ion Trap Mass Spectrometer instrument from Thermo Finnigan (San Josè, CA, USA) equipped with an electrospray ion source (ESI) and an Xcalibur® system manager data acquisition software. Sample solutions (5 μg ml^{-1}) were infused in the ESI source using a syringe pump at a flow rate of 2 μl ml^{-1} and the mass scan range was *m/z* 100–1000. Operating conditions on the ion trap mass spectrometer in negative polarity were as follows: spray voltage, 5.0 kV; source current, 80 μA; capillary temperature, 250 °C; capillary voltage, −45 V; tube lens offset, −60 V; multipole 1 offset, 6 V; multipole 2 offset, 10 V; sheath gas flow (N_2), 30 A.U. Data were acquired in MS, MS/MS and MS^n scanning mode and for all MS/MS and MS^n experiments the precursor isolation window was set at 1 atomic mass unit (a.m.u.) and the collision energy was optimized at 27–33%.

2.3. Liquid chromatography/electrospray mass spectrometry (LC–ESI-MS)

The extract was analyzed by LC–ESI-MS "on-line" using the same instrument described above connected to a Surveyor HPLC system (Thermo Finnigan, San Josè, CA, USA) equipped with a quaternary pump, a Surveyor AS autosampler and a vacuum degasser. The chromatographic separation was performed on a Symmetry Shield C-18 column (150 mm × 4.6 mm i.d., with particle size of 5 μm) (Waters Corporation, Milford, MA, USA) maintained at 35 °C. The solvents used as mobile phase were A: water, B: 1% formic acid in water (v/v) and C: acetonitrile. At the start of analysis, the composition was 76:1:23 (v/v/v). After 12-min hold in these conditions, elution was performed by a linear gradient from 76:1:23 to 60:1:39 in 8 min and then maintained for 5 min at a constant flow rate of 0.8 ml min^{-1}; the sample injection volume was 20 μl.

The eluate was injected into the electrospray ion source with a splitting of 20% and the MS and MS/MS spectra were acquired and interpreted using the software Xcalibur®.

2.4. Preparation of the stock solutions of cardenolides

A stock standard solution of k-strophanthoside was prepared in methanol at 1000 μg ml^{-1} and stored at +4 °C in the dark, where it found to be stable for at least 1 month. The solutions of the other standard glycosides were also done in methanol at 1000 μg ml^{-1} and identically stored. Work solutions were obtained just before use by appropriate dilutions in mobile phase water: formic acid 1% in water (v/v):acetonitrile, 76:1:23 (v/v/v).

2.5. Sample solutions

The solution of strophanthin-K sample was prepared in methanol at 1000 μg ml^{-1}. The dissolution of the sample was favoured by ultrasound treatment. Work solution was obtained just before use by dilutions in mobile phase water: formic acid 1% in water (v/v):acetonitrile, 76:1:23 (v/v/v), to a final concentration of 200 μg ml^{-1}.

2.6. Strophanthin-K degradation conditions

All degradation studies were done at a strophanthin-K concentration of 1000 μg ml^{-1}. For acidic decomposition studies, the sample was dissolved in 0.1 M HCl and solution was left in the dark at room temperature for 24 h. The studies in alkaline conditions were done in 0.1 M NaOH and solution was left in the dark at room temperature for 24 h. For oxidative conditions, studies were done in 3% hydrogen peroxide and solution was left in the dark at room temperature for 24 h. All the solutions were then diluted by adding an appropriate volume of mobile phase and analysed. For comparison purposes 10 mg of strophanthin-K were dissolved in 10 ml of methanol and diluted and analyzed as above.

3. Results and discussion

3.1. ESI-MS and ESI-MS/MS analysis of glycoside standards

Strophanthin-K was constituted by a mixture of cardiac glycosides, characterized by the same aglycone strophanthidin. K-strophanthoside was the main component being cymarin, helveticoside, erysimoside, k-strophanthin-β and gluco or neoglucoerysimoside the minor ones. Some of these were commercially available even if their purity was not stated. With the aim of obtaining mass spectrometric-based

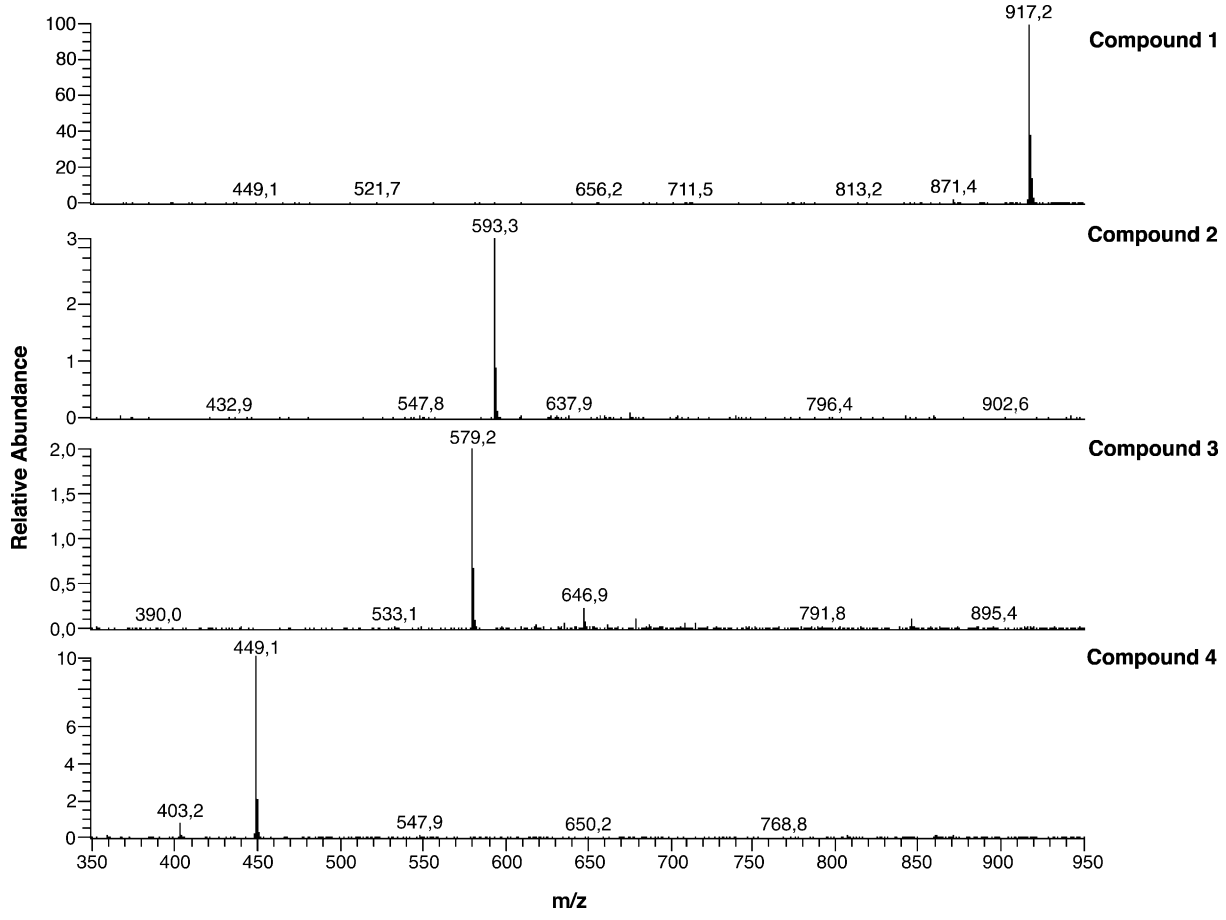

Fig. 2. ESI/MS spectra in negative ion mode of standard compounds **1**–**4**.

information focused to evaluate the presence of different glycosides in strophanthin-K, direct flow injection experiments of glycosides standards were initially performed.

In Fig. 2, the ESI-MS spectra in negative ion mode of k-strophanthoside, cymarin, helveticoside and strophanthidin were showed.

Formic acid enhanced the ionization efficiency of cardenolides in the ionic source. As reported for other cardiac glycosides such as digoxin, digitoxin and Lanatoside C [5] the formation of clusters was a typical feature of the ESI ionic source; indeed for all the compounds studied the more abundant ions in MS spectra in negative ion mode were not the pseudomolecular ions $[M-H]^-$ but formiate adducts $[M+HCOO]^-$.

To characterize the ionic fragmentation pattern of glycosides, MS/MS and MS^3 experiments were also performed and the data obtained from compounds **1**–**4** are shown in Table 1. The term "loss" was used when the glycosidic bond is cleaved between the anomeric carbon and the oxygen and "elimination" when the cleavage is between the glycosidic oxygen and the substituted carbon. Hence there is an 18 Da difference in the *m/z* values, corresponding to water, after the respective type of cleavage.

The daughter ion spectrum of the adduct ion at *m/z* 917 (compound **1**), 3-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 4)-β-D-cymarosopyranosyl strophanthidin, showed an intense pseudomolecular ion at *m/z* 871. In order to obtain more structural information, this ion was subjected to a further MS^3 fragmentation experiment.

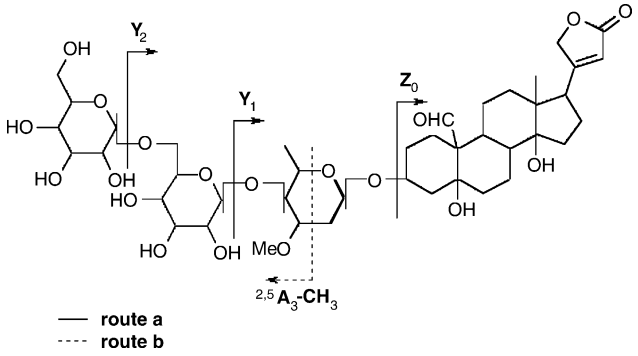

Fig. 3. Proposed scheme of fragmentation in negative ion mode MS^3 for pseudomolecular ion $[M-H]^-$ of compound **1** (k-strophanthoside).

The resulting spectra showed several structural specific fragments at *m/z* 709, 547, 409 and 385.

The formation of these ions could be explained according to the scheme reported in Fig. 3 where two parallel fragmentation routes are shown: route a—the ions formed on this route are in accordance to the Y_2, Y_1 and Z_0 series of fragmentation in polysaccharide chains [9] in terms of subsequent splitting of sugar unit from glycoside structure. Indeed, ions at *m/z* 709 and 547 arise from loss of one or two glucose units, respectively. Elimination of cymarose residue generated the ion at *m/z* 385 corresponding to the dehydrated aglycone ion. Route b—the intense fragment ion at *m/z* 409 is tentatively attributed to the carbohydrate moiety of the molecule as the result of the cleavage series $[^{2,5}A_3–CH_3]^-$ of cymarose unit

Table 1
Molecular ions and fragment ions of the glycosides, acquired by using ESI-MS on FIA mode (compounds **1**–**4**), and on LC–ESI-MS (compounds **5**–**7**)

Peak	Full MS (intensity)[a]	MS/MS (intensity)[a]	MS^3 (intensity)[a]	Correlated compound
A	903 $[M+HCOO]^-$ (100)	903 $[M+HCOO]^-$ parent ion (10) 857 $[M-H]^-$ (100)	857 $[M-H]^-$ parent ion (10) 695 $[M-H-162]^-$ (60) 533 $[M-H-162-162]^-$ (20) 409 $[M-H-448]^-$ (100) 385 $[M-H-162-162-148]^-$ (20)	**7**
B	917 $[M+HCOO]^-$ (100)	917 $[M+HCOO]^-$ parent ion (10) 871 $[M-H]^-$ (100)	871 $[M-H]^-$ parent ion (10) 709 $[M-H-162]^-$ (85) 547 $[M-H-162-162]^-$ (18) 409 $[M-H-462]^-$ (100) 385 $[M-H-162-162-162]^-$ (12)	**1**
C	741 $[M+HCOO]^-$ (100)	741 $[M+HCOO]^-$ parent ion (10) 695 $[M-H]^-$ (100)	695 $[M-H]^-$ parent ion (10) 533 $[M-H-162]^-$ (90) 385 $[M-H-162-148]^-$ (100)	**5**
D	755 $[M+HCOO]^-$ (100)	755 $[M+HCOO]^-$ parent ion (10) 709 $[M-H]^-$ (100)	709 $[M-H]^-$ parent ion (10) 547 $[M-H-162]^-$ (50) 385 $[M-H-162-162]^-$ (100)	**6**
E	449 $[M+HCOO]^-$ (80) 403 $[M-H]^-$ (20)	449 $[M+HCOO]^-$ parent ion (10) 403 $[M-H]^-$ (100)		**4**
F	579 $[M+HCOO]^-$ (100)	579 $[M+HCOO]^-$ parent ion (10) 533 $[M-H]^-$ (100)	533 $[M-H]^-$ parent ion (10) 385 $[M-H-148]^-$ (100)	**3**
G	593 $[M+HCOO]^-$ (100)	593 $[M+HCOO]^-$ parent ion (10) 547 $[M-H]^-$ (100)	547 $[M-H]^-$ parent ion (10) 385 $[M-H-162]^-$ (100)	**2**

[a] Normalized relative abundance.

with methyl transfer [9]. Ion at *m/z* 409 was further investigated by MS^4 analysis to support the suggested fragmentation route: the MS^4 spectrum (not shown) showed an intense ion at *m/z* 323 related to a diglucose structure, which confirmed the hypothesis.

On the basis of the fragmentation pattern elucidated from the standard k-strophanthoside, we expected that other cardiac glycosides contained in strophanthin-K would exhibit analogous behaviour (i.e. the production of dehydrated aglycone ion besides loss of sugar residues).

The MS, MS/MS and MS^3 analysis of compound **2,** 3-*O*-β-D-cymarosopyranosyl strophanthidin, and compound **3,** 3-*O*-4-β-D-digitoxopyranosyl strophanthidin, showed the same fragmentation pattern being the formiate adduct $[M + HCOO]^-$ the base peak ion in MS spectra and the pseudomolecular ion $[M - H]^-$ the base peak ion in MS/MS spectra. Moreover the dehydrated aglycone ion *at m/z* 385, corresponding to successive splitting of cymarose or digitoxose unit, respectively, was the base peak in MS^3 spectra. Differences in intensity of the ion fragments are summarized in Table 1.

Instead compound **4**, strophanthidin, the aglycone of all strophanthin-K glycosides, showed in MS spectra an intense ion at *m/z* 449 attributed to the adduct ion $[M + HCOO]^-$ and in MS/MS spectra the pseudomolecular ion $[M - H]^-$ at *m/z* 403.

Taken together, these data showed the usefulness of MS^3 experiments in negative ion mode to establish the number and the type of sugars attached to the aglycone moiety in glycoside characterization.

3.2. Liquid chromatography/electrospray mass spectrometry analysis of glycosides

To separate the cardiac glycosides contained in strophanthin-K a RP C-18 column was used. A gradient was found necessary to obtain an acceptable run time and an adequate resolution between the glycosides due to the large range of polarities exhibited by the different glycosides found in strophanthin-K. Fig. 4 presents the chromatogram obtained by injecting a strophanthin-K solution and collecting the data in full-scan negative ion mode and screening the mass-range of every peak analyzed. Seven main peaks (A–G) were detected and further analyzed by LC–ESI-MS/MS and LC–ESI-MS^n experiments.

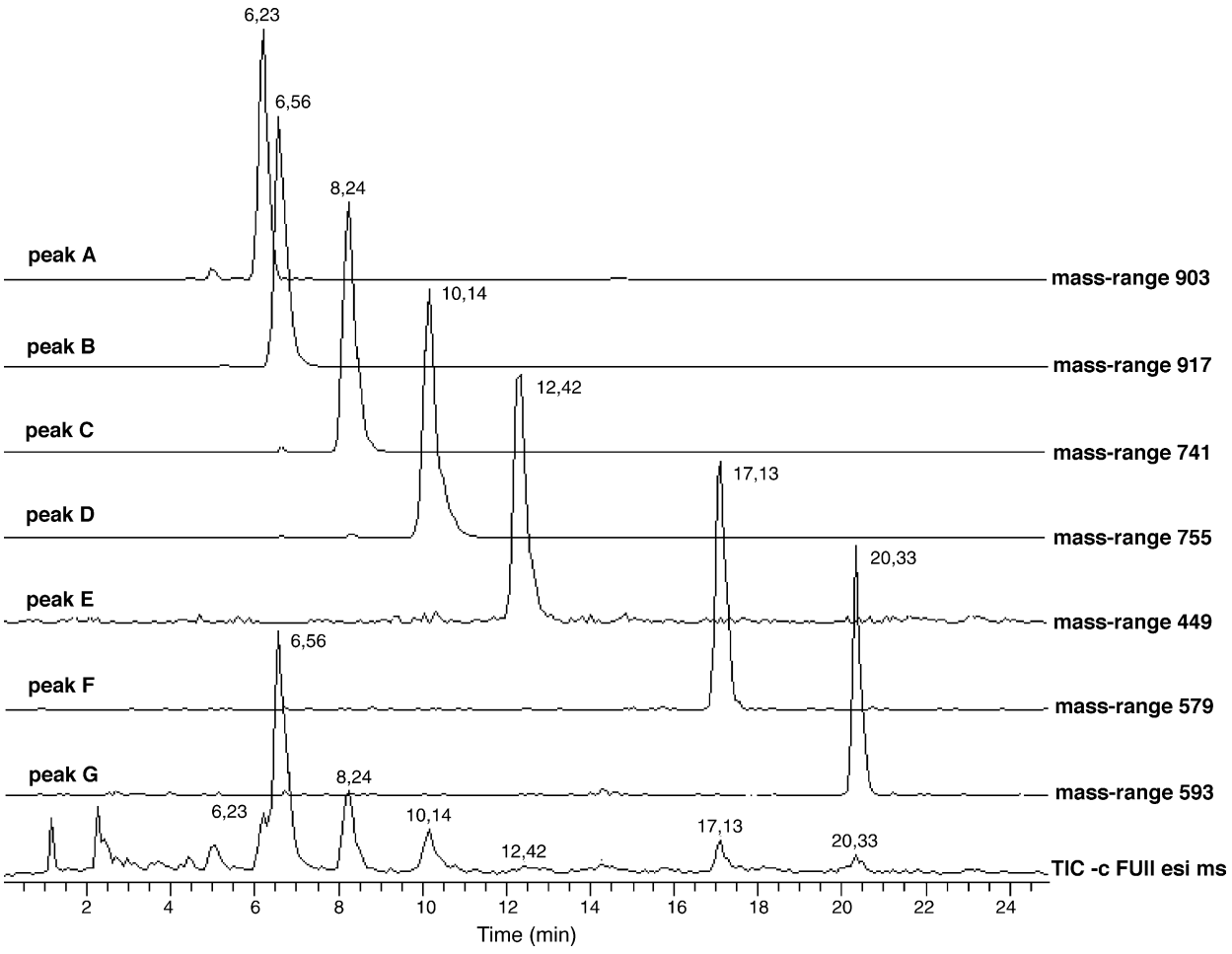

Fig. 4. Total negative ion current chromatogram of solution of strophanthin-K and the relative mass-range of each peak detected (A–G).

Negative ions mass spectra obtained from peaks B, E, F and G at retention time (t_R) 6.56, 12.42, 17.13 and 20.33 min as well as their *m/z* values were all in keeping to the structures of k-strophanthoside (compound **1**), strophanthidin (compound **4**), helveticoside (compound **3**) and cymarin (compound **2**). These assignments were further confirmed by comparing the retention time of the total ion chromatogram of the corresponding standard glycosides. Because of the lack of authentic samples the characterization of the structures of the peaks A, C and D at t_R 6.23, 8.24 and 10.14 min, respectively, was assigned basing only on mass spectral data as reported on Table 1. The evaluation of ion fragmentation pattern of negative ion spectra revealed the presence of the ion at *m/z* 385 corresponding to the dehydrated strophanthidin suggesting that peaks A, C and D were structurally related to the strophanthin-K glycosides.

In particular, for peak A at t_R 6.23 min MS^3 spectrum, arising from the pseudomolecular ion at *m/z* 857, showed three main ions at *m/z* 695, 533 and 385 that were generated respectively by subsequent losses of one or two glucose unit and elimination of a digitoxose unit according to the Y_2, Y_1, Z_0 series of fragmentation in polysaccharide chain [9]. Moreover, MS^3 spectrum showed an intense ion at *m/z* 409 confirming the presence of three-sugar unit as for k-strophanthoside. Based on these data the structure of 3-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 4)-β-D-digitoxopyranosyl strophanthidin was assigned to peak A (compound **7**). This compound was regarded in agreement with neoglucoerysimoside as reported by Makarevich [4], where the interglycosidic linkages were determined to be (1 → 6)-(1 → 4) as the analogous trisaccharide glycoside k-strophanthoside. On the other hand, recently [10], the name glucoerysimoside (3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-β-D-digitoxopyranosyl strophanthidin) was instead attributed to a glycosidic compound of *Erysimum cheirathoides*, a plant belonging to a Cruciferae family, and characterized by two glucose and one digitoxose units linked to strophanthidin aglycone where the interglycosidic linkages were determined to be (1 → 4)-(1 → 4).

Peak C at t_R 8.24 min presented in full scan mass spectrum an ion at *m/z* 741 corresponding to formiate adduct, which generates in MS/MS spectrum the corresponding pseudomolecular ion $[M-H]^-$ at *m/z* 695. The ion frag-

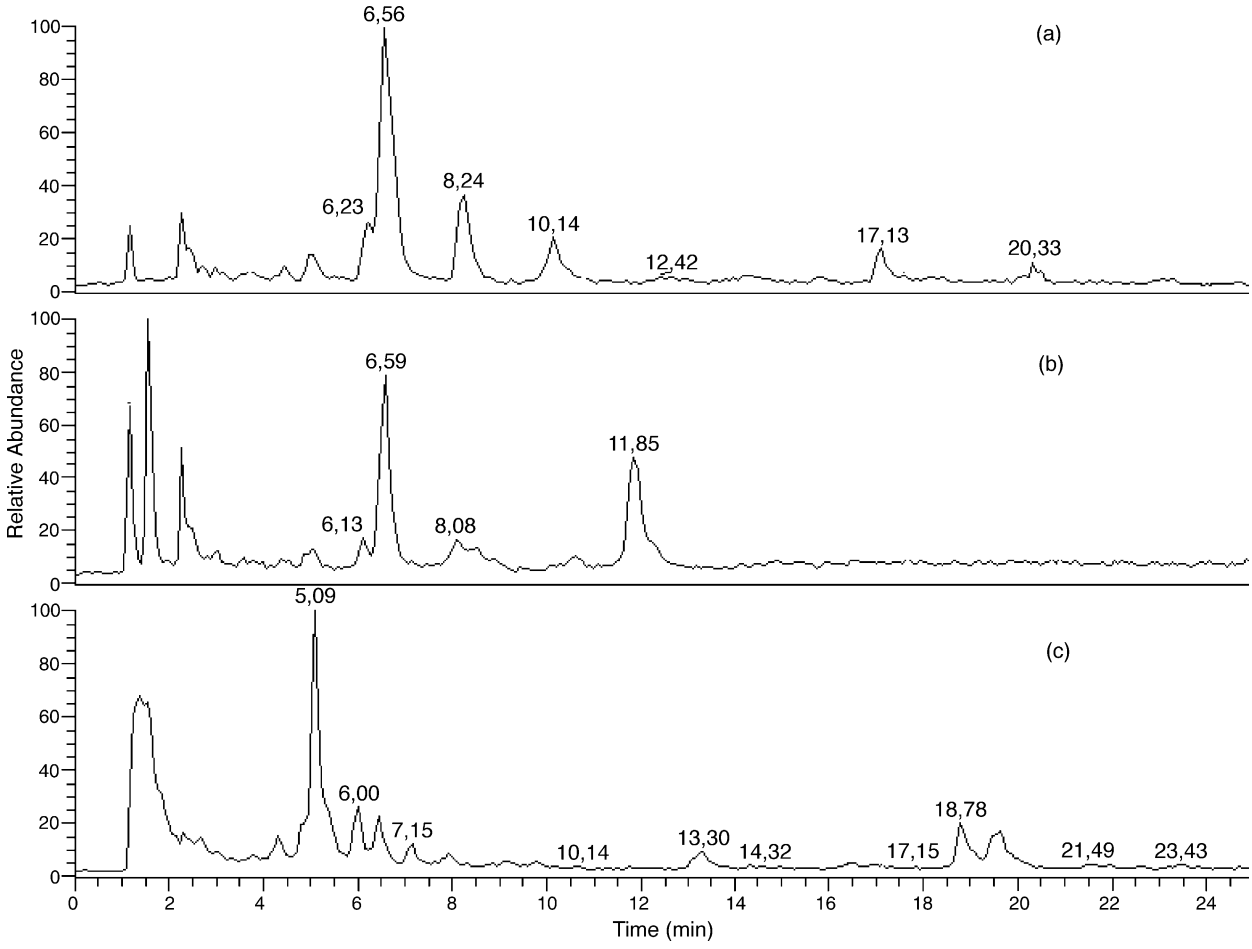

Fig. 5. Representative LC–MS chromatogram of strophanthin-K. Key: standard solution (a), sample degraded in 0.1 M HCl (b), sample degraded in 0.1 M NaOH (c).

Table 2
Comparison between retention time (t_R, min) and molecular weight (MW) of glycosides and glycosides γ-hydroxy acids obtained under alkaline degradation.

Peak[a]	Compound	t_R (min)[a]	t_R (min)[b]	MW[a]	MW[b]	$[M-H]^-$ (m/z)[b]
A	**7**	6.23	4.88	858	876	875
B	**1**	6.56	5.09	872	890	889
C	**5**	8.24	6.00	696	714	713
D	**6**	10.14	7.15	710	728	727
F	**3**	17.13	13.30	534	552	551
G	**2**	20.33	18.78	548	566	565

[a] Before degradation.
[b] After degradation.

ments, at *m/z* 533 and 385, obtained in MS^3 experiments, arose from the loss of a glucopyranose and elimination of digitoxose units, respectively. These data allowed to assign the structure of 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-digitoxopyranosyl strophanthidin (erysimoside) to peak C (compound **5**).

Peak D at t_R 10.14 min presented in full scan mass spectrum an ion at *m/z* 755 corresponding to formiate adduct, which generates in MS/MS spectrum the corresponding pseudomolecular ion $(M-H)^-$ at *m/z* 709. The ion fragments, at *m/z* 547 and 385, obtained in MS^3 experiments, arose from the loss of a glucopyranose and elimination of cymarose units, respectively. These data allowed to assign the structure of 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-cymarosopyranosyl strophanthidin (k-strophanthin-β) to peak D (compound **6**).

Interestingly, only the glycosides with three sugars unit (k-strophanthoside and neoglucoerysimoside) showed in the MS^3 spectra an intense ion at *m/z* 409 due to the carbohydrate moiety, while mono and diglycosides showed in the MS^3 spectra only ions that retain the charge on the aglycone side of the molecule.

3.3. Degradation products of strophanthin-K

As expected from their structural features, strophanthin-K glycosides have similar chemical properties and stability of other cardenolides [11]. Hence, the glycoside links and the unsaturated lactone ring should be the labile moiety involved in hydrolytic degradation reactions. LC–MS studies on strophanthin-K under acidic, alkaline and oxidative conditions established the following degradation behaviour:

3.3.1. Acidic conditions

On letting strophanthin-K in 0.1 M HCl for 24 h at room temperature, around 90% degradation was seen. As reported in Fig. 5b the peak corresponding to k-strophanthoside (t_R 6.56 min) was reduced and that one corresponding to aglycone strophanthidin (t_R 11.85 min) was increased as a consequence of the cleavage of the glycosidic linkage in acidic conditions. The same behaviour was observed for all the glycosides present in strophanthin-K sample.

3.3.2. Alkaline conditions

Strophanthin-K was found to be labile in alkali. Sample degradation of the compound was observed in 0.1 M NaOH at room temperature for 24 h. As shown in the chromatogram (Fig. 5c), one major degradation product is formed at t_R lower than that of k-strophanthoside in standard solution (Fig. 5a). The ESI-MS spectra is in agreement with a molecular weight of 890, 18 a.m.u. more than k-strophanthoside that was explained with the hydrolysis of the unsaturated 17β-lactone of the aglycone moiety to the corresponding γ-hydroxy acid. Analogously, all glycosides present in strophanthin-K sample, under alkaline degradation underwent to aglycone moiety hydrolysis generating the correspondent γ-hydroxy acids. In Table 2, t_R and MW of glycosides and γ-hydroxy acids are reported in comparison, as well as the pseudomolecular ions $[M-H]^-$ observed in LC–ESI/MS spectra in negative ion mode of the alkaline degraded sample.

3.3.3. Oxidative conditions

Strophanthin-K extract was found to be practically stable in 3% H_2O_2 at room temperature for 24 hours.

4. Conclusions

In this study, an HPLC–ESI-MS/MS method has been developed for the characterization of the cardiac glycosides of strophanthin-K extract from *Strophanthus kombé*.

MS^n spectra in negative ion mode were an excellent tool to characterize the sequence of sugars in glycosides with strophanthidin as aglycone moiety. Moreover, the specificity of the base peak in MS^3 spectrum of k-strophanthoside, the main glycoside present in strophanthin-K extract, could be a useful tool for a rapid and sensitive detection of these class of cardenolides.

Strophanthin-K was also studied in stressed conditions: it was found to degrade in alkaline and acidic conditions while was practically stable in oxidative conditions.

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