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Chromatographic and mass spectral characterization of budesonide and a series of structurally related corticosteroids using LC–MS

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Abstract

The LC–MS characteristics of budesonide and a series of structurally related corticosteroids were reviewed to commence the construction of a library of chromatographic and mass spectral information to aid identification of budesonide degradation products during formulation stabilization investigations. The LC–ESI$^+$–MS technique employing a Hypersil® C18 column with a mobile phase of ethanol-acetonitrile-formic acid (pH 3.8; 0.14 mM) (2:30:68, v/v/v) was then used to characterize 23 corticosteroids. Based on their structures, the corticosteroids were classified into three groups: (I) 4-pregnene-3-one steroids; (II) 1,4-pregnadien-3-one steroids with no fluorine substituents; and (III) 1,4-pregnadiene-3-one steroids with fluorine substituents. Chromatographic (retention time and UV absorbance) and mass spectral properties were correlated with the known chemical structures of these corticosteroids. Base peak and mass spectral fragmentation patterns were related to steroid structural characteristics.

Keywords: LC–MS; Corticosteroid; Budesonide; Database; Degradation; Stabilization

1. Introduction

Mass spectrometry has been widely employed to characterize a diverse range of corticosteroids. It has been used mainly in support of medicinal chemistry and drug discovery efforts, aiding the structural elucidation of novel compounds. Zaretskii [1] and Budzikiewicz [2,3] systematically reviewed the mass spectral characteristics of a series of steroids using electroionization (EI) and chemical ionization (CI) techniques. Kobayashi et al. [4] studied the mass spectra of sixty steroids using LC–MS with APCI and concluded that it was a suitable technique to deduce molecular weight and structural information. They also observed that ionization intensities and mass spectral characteristics were affected by ionization parameters such as nebulizer temperature and drift voltages. However, despite the widespread use of MS and LC–MS technologies for the analysis of corticosteroids, their use in the identification of steroid pharmaceutical degradation products has been neglected.

The 4-pregnene-3-one and the 1,4-pregnandien-3-one corticosteroids are anti-inflammatory drugs widely used for systemic and topical treatment of different diseases, such as inflammation, allergies, asthma and arthritis. Budesonide, a 1,4-pregnadien-3-one, is a relatively new steroid currently being developed and used in new formulations for aerosol drug delivery, both in metered dose inhalers and with novel condensation aerosol technology [5,6]. Accordingly, the stabilization of these steroids in non-aqueous solutions has become important. A number of recent patent applications have focused on stabilizing these formulations using anti-oxidants and canister protective coatings [7–9]. Previously published stability studies of hydrocortisone and prednisolone revealed numerous complex degradation pathways [10–12]. Stability of those compounds was affected by a variety of factors, including the presence of air, pH, metal ions, solvent composition, pharmaceutical additives and light [13–15]. However, the majority of these studies were performed in

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the 1970s and early 1980s, without the benefit of online LC–MS technologies and the identification of the degradation products were limited. Recently, Wu et al. and Rehfuss et al. used LC–MS to propose the identity of major ethanolic solution degradation products of triamcinolone acetonide and budesonide; however, no details of the LC–MS method were presented [16–17]. Electron impact fragmentation MS has been used to investigate the mass spectra of budesonide epimers and related synthetic analogues during drug development [18]. In addition, numerous clinical studies have employed LC–MS for elucidation of budesonide metabolic pathways and quantification of budesonide [19–21].

In this paper, we review the ionization characteristics of budesonide and present an LC–MS method that was used to qualitatively characterize the chromatographic and mass spectral properties of budesonide and a series of structurally related corticosteroids selected in order to aid in the identification of budesonide breakdown products. The relationship between known structural functional groups and mass spectral fragmentation patterns were investigated as functions of increasing in-source collision-induced dissociation. This study generated a library of information that was employed to aid the identification of degradation products generated during a series of accelerated stability studies using budesonide-in-propylene glycol solutions [22].

2. Experimental

2.1. Materials

Steroids were purchased from Sigma Chemical Co. (St. Louis, MO), Spectrum Quality Products Inc. (New Brunswick, NJ) and Steraloids Inc. (Newport, RI), respectively. Methanol, ethanol, acetonitrile and formic acid 88% were purchased from Fisher Scientific Co. (Swannee, GA). All these reagents were HPLC grade.

2.2. Instrumentation

The HPLC system consisted of Waters Alliance 2690 separations module and a Waters 996 photodiode array (PDA) UV detector (Waters Corp., Milford, MA). A Micromass ZMD4000 single quadrupole mass spectrometer with ESI ionization probe was used (Waters Corp., Milford, MA).

2.3. LC–MS conditions

A Hypersil C18 column (150 mm × 4.6 mm i.d., 5 μm particle) was employed and ethanol-acetonitrile-formic acid (pH 3.8; 0.14 mM) (2:30:68, v/v/v) used as mobile phase at a flow rate of 1.5 mL/min. This method was adapted from a method previously developed as a quantitative HPLC-UV assay for budesonide [23]. A flow splitter (Upchurch Scientific Inc., Oak Harbor, WA) was used after the HPLC column so that approximately 1.2 mL/min of the eluent was delivered to the PDA detector, and about 0.3 mL/min introduced to the electrospray probe of the mass spectrometer. UV chromatograms were obtained from the PDA detector, with the wavelength range set to scan from 200 to 400 nm. For the MS ionization conditions, the source block temperature and the desolvation temperature were held at 150 and 225 °C, respectively. Capillary and cone voltages of 4.0 kV and 25 V were selected following optimization. The desolvation nitrogen flow was 700 L/h. In order to induce fragmentation by collision-induced disassociation (CID), many analyses were repeated using a cone voltage of 50 V, while the remaining parameters were held constant. Mass spectra were acquired in the mass range from 90 to 1000 Da at 400 amu/s.

Based on their structures, 23 corticosteroids, of which budesonide was one, were acquired and subdivided into three groups: (I) 4-pregnene-3-one corticosteroids; (II) 1,4-pregnadien-3-one corticosteroids with no fluorine substituents; and (III) 1,4-pregnadine-3-one corticosteroids with fluorine substituents. These are shown in Fig. 1 and Tables 1–3. Standard stock solutions (0.1 through 0.7 mM) were prepared for each corticosteroid by dissolving the substance directly in methanol. Rather than analyzing each steroid individually, mixtures were prepared. 200 μL of each Group I steroid solution were combined to produce mixture solution A. Due to the co-elution of some corticosteroids in Group II, two mixture solutions were prepared for this group of corticosteroids (mixture solution Bi and Bii). Mixture solution C was obtained for Group III corticosteroids following similar procedures. As an internal reference budesonide was included in each of these mixtures. Twenty microlitres of each mixture was injected.

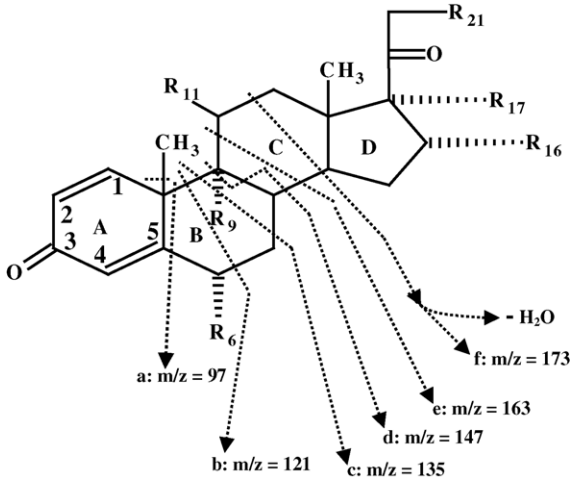
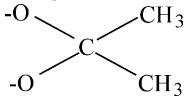
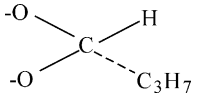
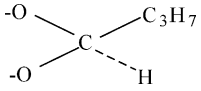


Fig. 1. Structure skeleton of 1,4-pregnadien-3-one corticosteroids. Dashed lines (a–f) show possible fragmentation pathways discussed in the text. The complete structures of the R- and S-epimers of budesonide are discussed in Table 2.

Table 1
Functional groups of the 4-pregnene-3-one corticosteroids shown in Fig. 1 (Group I)

Steroids	$\Delta^{1,2}$	R_6	R_9	R_{11}	R_{16}	R_{17}	R_{21}
Hydrocortisone	Saturated	–H	–H	–OH	–H	–OH	–OH
Cortisone	Saturated	–H	–H	=O	–H	–OH	–OH
11-Dehydrocorticosterone	Saturated	–H	–H	=O	–H	–H	–OH
Corticosterone	Saturated	–H	–H	–OH	–H	–H	–OH
Reichestin's substance S	Saturated	–H	–H	–H	–H	–OH	–OH
21-Deoxycortisone	Saturated	–H	–H	=O	–H	–OH	–H
Deoxycorticosterone	Saturated	–H	–H	–H	–H	–H	–OH

Table 2
Functional groups of the 1,4-pregnadien-3-one corticosteroids shown in Fig. 1 with no fluorine substituents (Group II)

Steroids	$\Delta^{1,2}$	R_6	R_9	R_{11}	R_{16}	R_{17}	R_{21}
Prednisolone	Double bond	–H	–H	–OH	–H	–OH	–OH
Prednisone	Double bond	–H	–H	=O	–H	–OH	–OH
Methylprednisolone	Double bond	$-CH_3$	–H	–OH	–H	–OH	–OH
1,4-Pregnadien-17,21-diol-3,20-dione	Double bond	–H	–H	–H	–H	–OH	–OH
1,4-Pregnadien-6α-methyl-17,21-diol-3,11,20-trione	Double bond	$-CH_3$	–H	=O	–H	–OH	–OH
1,4-Pregnadien-l1β,21-diol-3,20-dione	Double bond	–H	–H	–OH	–H	–H	–OH
1,4-Pregnadien-16β-methyl-17,21-diol-3,11,20-trione	Double bond	–H	–H	=O	$-CH_3$	–OH	–OH
Desonide	Double bond	–H	–H	–OH			–OH
Prednisolone acetate	Double bond	–H	–H	–OH	–H	–OH	$-OCOCH_3$
1,4-Pregnadien-6α-methyl-11β,17-diol-3,20-dione	Double bond	$-CH_3$	–H	–OH	–H	–OH	–H
1,4-Pregnadien-21-ol-3,20-dione	Double bond	–H	–H	–H	–H	–H	–OH
Budesonide R-epimer	Double bond	–H	–H	–OH			–OH
Budesonide S-epimer	Double bond	–H	–H	–OH			–OH

3. Results and discussion

3.1. HPLC chromatograms of corticosteroids

The HPLC chromatographic parameters for the 23 corticosteroids are summarized in Table 4. Fig. 2 shows a typical HPLC-UV chromatogram for the steroids in Group I. Using this isocratic HPLC method, all these 23 corticosteroids could be eluted within 25 min. In general, a number of effects on retention time were observed across each group of compounds (Tables 1–3) due to structural and functional group changes. The introduction of a $\Delta^{1,2}$ double bond, changing from a 4-pregnene-3-one corticosteroids (Group I) to a 1,4-pregnadien-3-one (Group II) decreased the retention times of the corticosteroid. For example, deoxycorticosterone (Group I) had a retention time of 17.8 min. However, 1,4-pregnadien-21-ol-3,20-dione (Group II), with an identical structure except for its $\Delta^{1,2}$ double bond, had a retention time of 10.8 min. As expected, the presence of additional hydroxyl groups (–OH) decreased retention times, due to

Table 3
Functional groups of the 1,4-pregnadiene-3-one corticosteroids shown in Fig. 1 with fluorine substituents (Group III)

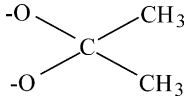
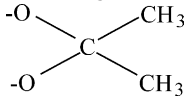
Steroids	$\Delta^{1,2}$	R_6	R_9	R_{11}	R_{16}	R_{17}	R_{21}
Triamcinolone	Double bond	–H	–F	–OH	–OH	–OH	–OH
Triamcinolone acetonide	Double bond	–H	–F	–OH			–OH
Triamcinolone diacetate	Double bond	–H	–F	–OH	$-OCOCH_3$	–OH	$-OCOCH_3$
Fluocinolone acetonide	Double bond	–F	–F	–OH			–OH

Table 4
The retention times and maximum wavelengths for absorption (λ_{max}) for corticosteroids during HPLC assay

Name	Maximum wavelength λ_{max} (nm)	Retention time (min)
Group I		
Hydrocortisone	247	3.4
Cortisone	244	3.6
11-Dehydrocorticosterone	244	5.5
Corticosterone	248	6.6
Reichesteins's substance S	248	7.5
21-Deoxycortisone	244	8.5
Deoxycorticosterone	248	17.8
Group II		
Prednisolone	247	3.2
Prednisone	242	3.3
Methylprednisolone	248	5.1
1,4-Pregnadien-17,21-diol-3,20-dione	249	5.2
1,4-Pregnadien-6α-methyl-17,21-diol-3,11,20-trione	243	5.3
1,4-Pregnadien-11β,21-diol-3,20-dione	248	5.8
1,4-Pregnadien-16β-methyl-17,21-diol-3,11,20-trione	242	5.8
Desonide	248	7.2
Prednisolone acetate	248	9.3
1,4-Pregnadien-6α-methyl-11β,17-diol-3,20-dione	248	10.4
1,4-Pregnadien-21-ol-3,20-dione	249	10.8
R-budesonide	248	18.9
S-budesonide	248	20.9
Group III		
Triamcinolone	242	1.6
Triamcinolone acetonide	242	7.5
Triamcinolone diaceatate	242	9.1
Fluocinolone acetate	242	9.1

increasing analyte polarity. For example, deoxycorticosterone (Table 1) eluted at 17.8 min, while corticosterone (–OH at C-11), Reichestein's substance S (–OH at C-17) and hydrocortisone (–OH at both C-11 and C-17) eluted at 6.6, 7.5, and 3.4 min, respectively. Compared to desonide's (Table 2) retention time of 7.2 min, triamcinolone acetonide (Table 3; –F at C-9) and fluocinolone acetonide (Table 3; –F at both C-6 and C-9) had retention times of 7.5 min and 9.1 min, suggesting that the presence of electronegative fluorine atoms at C-6 and C-9 marginally increased these retention times of corticosteroids. The presence of an acetal side chain at C-16 and C-17 also increased the retention times. For example, desonide and both of budesonide's R- and S-epimers had retention times of 7.2, 18.9 and 20.9 min, respectively. However, prednisolone, without these C-16 and C-17 substituents, had a retention time of 3.2 min. Similarly, triamcinolone and triamcinolone acetonide eluted at 1.6 and 7.5 min, respectively. Wikby et al. reported that using a reversed-phase HPLC, the retention time for corticosteroids with C-16 and C-17 cyclic acetal side chains was proportional to the length of the acetal side chain [24].

The maximum UV absorption wavelength (λ_{max}) for each of the corticosteroids is also shown in Table 4 to aid in identification. Most of the corticosteroids had λ_{max} of 248 nm and the introduction of a $\Delta^{1,2}$ double bond, the addition of an acetal side chain at C-16 and C-17 and the presence of hydroxyl groups at C-11 and/or C-17 had little affect. However, the presence of a carbonyl substituent (C=O) at C-11 had significant hypsochromic effects for both 4-pregnene-3-one corticosteroids (e.g., cortisone: λ_{max} = 244 nm) and for 1,4-pregnadien-3-one corticosteroids (e.g., prednisone: λ_{max} = 242 nm). This was also the case for the presence of fluorine atoms at C-6 and/or C-9, so that λ_{max} for all Group III corticosteroids was 242 nm. These results were consistent with the UV absorption characteristics of steroids as reviewed by Dusza et al. [25].

3.2. Mass spectra of budesonide

Mass spectra for the R-epimer of budesonide obtained during optimized LC–MS (Section 2.3) are shown in Fig. 3 at two cone voltages. Identical results were obtained for the S-epimer. At a cone voltage of 25 V (Fig. 3a), three categories

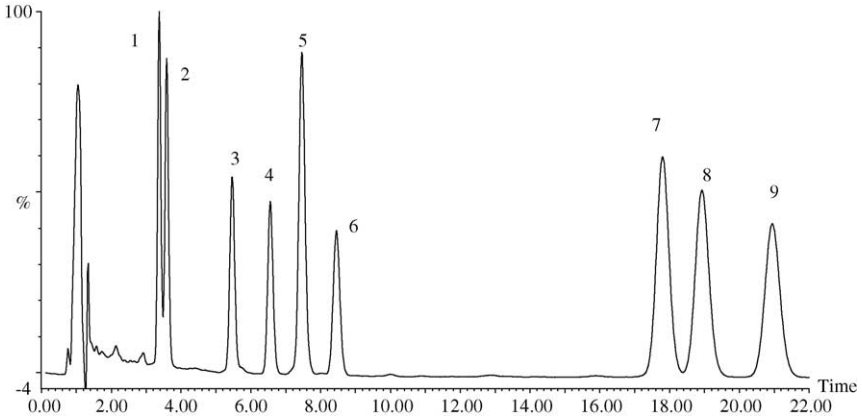


Fig. 2. Typical HPLC-UV chromatogram for the Group I steroids: (1) hydrocortisone; (2) cortisone; (3) 11-dehydrocorticosterone; (4) corticosterone; (5) reichestin's substance S; (6) 21-desoxycortisone; (7) deoxycorticosterone; (8) R-budesonide; (9) S-budesonide.

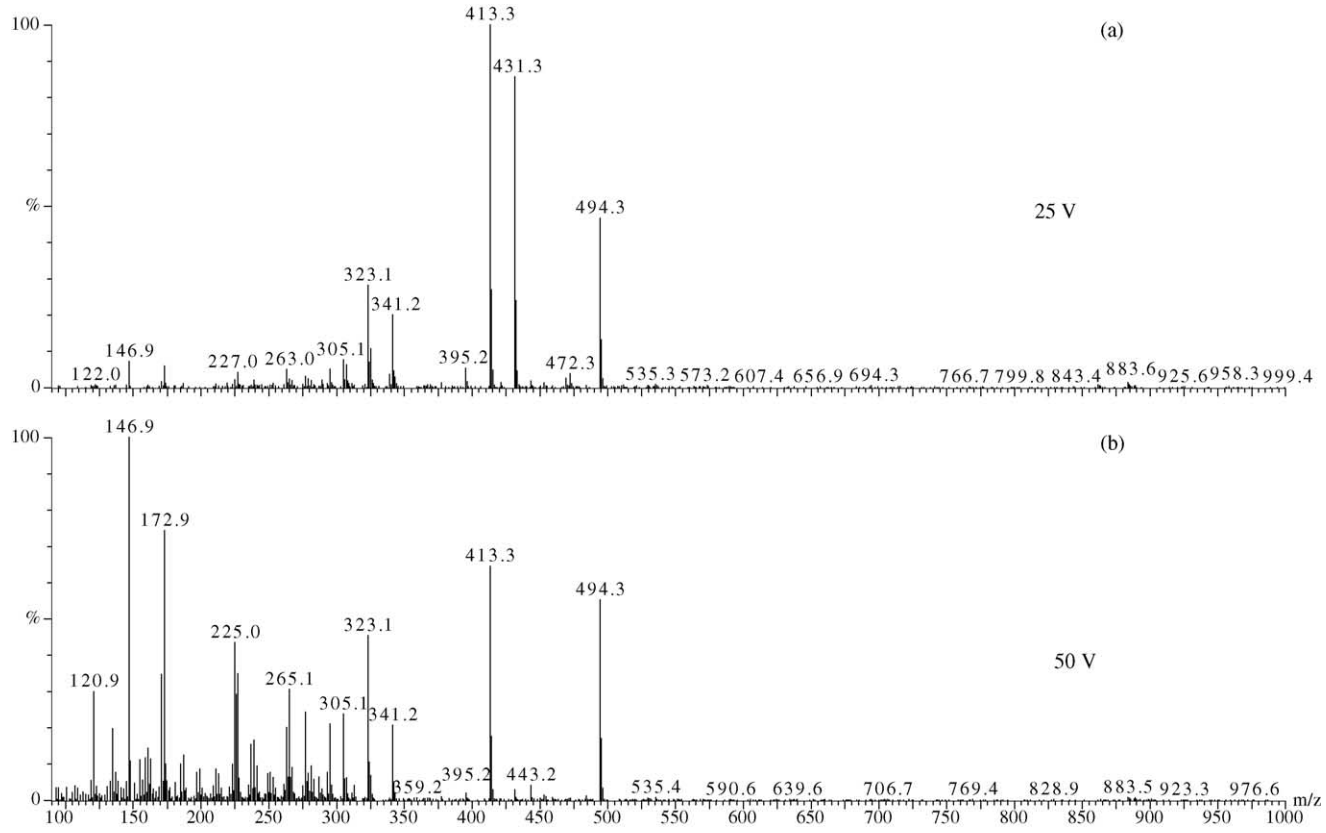


Fig. 3. Mass spectra of the R-epimer of budesonide obtained from optimized LC–MS according to Section 2.3. The cone voltage was (a) 25 V and (b) 50 V.

of ions were observed in the mass spectra: the parent ion ($[M + H]^+$, $m/z = 431$), adducts such as $[M + Na + CH_3CN]^+$ ($m/z = 494$), and fragments such as $m/z = 413$, 341, 323, and 147. The dehydration fragment ($[M + H - H_2O]^+$, $m/z = 413$) was observed as the base peak at a cone voltage of 25 V, however, the parent ion ($[M + H]^+$) was still present at a relatively high intensity. When the cone voltage was increased to 50 V (Fig. 3b), the $[M + H]^+$ peak almost disappeared and high intensity low mass fragments were observed with the $m/z = 147$ ion peak as the base peak.

3.3. Characteristics of corticosteroid mass spectra at a cone voltage of 25 V

Using a cone voltage of 25 V, three types of ion were observed in the mass spectra: (1) protonated molecular ion ($[M + H]^+$); (2) fragments, such as $[M + H - H_2O]^+$ and $[M + H - 2H_2O]^+$; and (3) adducts, such as $[M + H + CH_3CN]^+$ and $[M + Na + CH_3CN]^+$. Tables 5–7 summarize the major ions (defined as relative peak intensity $\geq$10% with respect to the base peak) observed in the mass spectra of each group of corticosteroids. These characteristics are discussed briefly below.

3.3.1. Group I, 4-pregnene-3-one corticosteroids

The major ions for Group I corticosteroids are shown in Table 5. $[M + H]^+$ was the base peak for all these corticosteroids. Little fragmentation was observed in their mass spectra, which suggested the relative stability of $[M + H]^+$ ions under these conditions. The predominant adduct for the Group I corticosteroids was $[M + H + CH_3CN]^+$. The adduct $[M + Na + CH_3CN]^+$ was only formed for hydrocortisone and corticosterone, which both had –OH at C-11 implicating this group in the formation of this complex.

3.3.2. Group II, 1,4-pregnadien-3-one corticosteroids without fluorine substituents

The introduction of a $\Delta^{1,2}$ double bond in the structures of Group II steroids produced significant differences in the mass spectra compared to Group I steroids. Significant fragmentation at the lower cone voltage of 25 V was observed, the main fragments being dehydration products (e.g., $[M + H - H_2O]^+$) (Table 6). Identity of the base peak was mainly dependent upon the substituent group at the C-11 position. When a hydroxyl group (–OH) was present at the C-11 position, e.g., budesonide (Fig. 3a) and prednisolone, the dehydration fragment ($[M + H - H_2O]^+$) formed the base peak. Also present in these mass spectra were consecutive dehydration fragments (e.g., $[M + H - 2H_2O]^+$ and $[M + H - 3H_2O]^+$). Clearly, the –OH group at C-11 was easily lost during ionization and the $[M + H - H_2O]^+$ ion was produced subsequently. When there were no substituents or a carbonyl group at C-11, e.g., prednisone, the parent ion ($[M + H]^+$) formed the base peak. However, de-

Table 5
Major ions in mass spectra of Group I corticosteroids at cone voltage = 25 V

Steroids	M.W.	$[M+H]^+$	$[M+H+CH_3CN]^+$	$[M+Na+CH_3CN]^+$	Base peak at 50 V
Hydrocortisone	362.5	**363.2 (100)**	404.2 (18)	426.2 (18)	**120.8**
Cortisone	360.4	**361.1 (100)**	402.3 (32)		**162.9**
11-Dehydrocorticosterone	344.4	**345.1 (100)**	386.2 (39)		**345.1**
Corticosterone	346.5	**347.2 (100)**	388.2 (23)	410.2 (18)	**120.8**
Reichesteins's substance S	346.5	**347.2 (100)**	388.2 (29)		**96.9**
21-Deoxycortisone	344.4	**345.1 (100)**	386.2 (32)		**345.1**
Deoxycorticosterone	330.5	**331.2 (100)**	372.2 (30)		**96.9**

Numbers in parentheses represent the relative peak intensity with respect to the base peak. The base peaks at cone voltage = 50 V are also shown. Base peaks are shown in bold font.

hydration fragments were also observed in the mass spectra of Group II corticosteroids, a result which contrasted with that from Group I steroids. Overall therefore, introduction of the $\Delta^{1,2}$ double bond decreased the stability of the $[M+H]^+$ ions. $[M+Na+CH_3CN]^+$ was formed as the major adduct (Table 6).

Furthermore, for budesonide and desonide with an acetal side chain at C-16 and C-17, two unique mass spectral fragments were observed (Fig. 3a; m/z = 341 and 323). These appeared to coincide with the loss of the acetal side chain (C_4H_8O, 72 amu) and one/two water molecules (H_2O, 18 amu), respectively. Notably, budesonide and desonide showed little further dehydration unlike other 1,4-pregnadien-3-one corticosteroids with –OH at C-11, such as prednisolone (Table 6). This implied that the introduction of the acetal side chain at C-16 and C-17 decreased the possibility of consecutive loss of water molecules perhaps due to steric hindrance at C-16 and C-17.

3.3.3. Group III, 1,4-pregnadien-3-one corticosteroids with fluorine substituents

Mass spectral ionization patterns are summarized in Table 7. Ionization at a cone voltage of 25 V resulted in the loss of the fluorine atom and $[M+H-HF]^+$ was the base peak in the mass spectra of both triamcinolone and triamcinolone acetonide. Dehydration fragments accompanying the loss of the fluorine atom (e.g., $[M+H-HF-H_2O]^+$) were also observed. Interestingly, when fluorine atoms appeared at both C-6 and C-9, e.g., fluocinolone acetonide $[M+H]^+$, formed as the base peak, even through fragments due to the loss of the fluorine atoms ($[M+H-HF]^+$ and $[M+H-2HF]^+$) were also produced. There was some evidence that the presence of fluorine at C-6 may impede the loss of fluorine at C-9, as well as the loss of water molecules from –OH at C-11, thus stabilizing the $[M+H]^+$ ions. Similar to desonide and budesonide ESI mass spectra, fragments formed following the loss of the acetal side chain at C-16 and C-17 plus water molecules followed the loss of fluorine atom(s) for triamcinolone acetonide and fluocinolone acetonide. The adduct $[M+Na]^+$ was observed as the base peak in triamcinolone diacetate's mass spectrum while the parent ion ($[M+H]^+$) and fragments of $[M+H-HF]^+$ (m/z = 459) and $[M+H-HF-H_2O]^+$ (m/z = 441) were also observed with high intensity. Similar to Group II corticosteroids, the formation of the $[M+Na+CH_3CN]^+$ adduct was favored in all Group III compounds (Table 7).

3.4. Characteristics of mass spectra at a cone voltage of 50 V

Increasing the cone voltage increased the molecular fragmentation for all corticosteroids, due presumably to increase in source collision induced disassociation (CID). As a result, the technique enabled more detailed structural information to be deduced, mainly concerning the substituents at C-6, C-11, C-17 and C-21 and the structural integrity of the A-ring.

3.4.1. Group I, 4-pregnene-3-one corticosteroids

The protonated molecular ions ($[M+H]^+$) were still observed at relatively high intensities (ranging 45%–99.9% with respect to the base peak), supporting the stability of $[M+H]^+$ ions in these cases. However, low molecular weight fragments mainly appeared as base peaks (Fig. 4), and their intensity was dependent on the substituent groups at the C-11, C-17 and C-21, as shown in Scheme 1.

While no substitutes were present on C-11, e.g., Reichestein's substance S (Fig. 4a) and deoxycorticosterone, the fragment m/z = 97 was observed as the base peak in the mass spectra, accompanied with the presence of another high intensity fragment of m/z = 109. For C-11 hydroxylated hydrocortisone (Fig. 4b) and corticosterone, the fragment m/z = 121 formed the base peak. Hydrocortisone and corticosterone also favored the formation of intermediate dehydration fragments. When a carbonyl group was present at C-11, the base peak appeared to be determined by the substituents at C-17 and C-21. For example, 11-dehydrocorticosterone (–OH at C-21, Fig. 4c) and 21-deoxycortisone (–OH at C-17, Fig. 4d) had [M + H]+ ions as their base peaks. For 21-deoxycortisone however, fragment m/z = 163 was also produced at a relatively high intensity (77%); fragment m/z = 285 was believed to be formed due to the loss of water and the $-COCH_3$ side chain formed at C-17. When both C-17 and C-21 were substituted by –OH groups (e.g., cortisone; Fig. 4e), the m/z = 163 fragment formed the base peak.

Table 6
Major ions in mass spectra of Group II corticosteroids at cone voltage = 25 V

Steroids	M.W.	$[M+H]^+$	$[M+H+CH_3CN]^+$	$[M+Na+CH_3CN]^+$	$[M+H-H_2O]^+$	$[M+H-2H_2O]^+$	$[M+H-3H_2O]^+$	Base peak at 50 V
Prednisolone	360.4	361.2 (85)		424.2 (58)	**343.2 (100)**	325.1 (38)	307.1 (17)	**146.9**
Prednisone	358.4	**359.2 (100)**		422.2 (30)	341.1 (47)			**146.9**
Methylprednisolone	374.5	375.1 (88)	416.1 (13)	438.1 (89)	**357.1 (100)**	339.0 (43)	321.0 (16)	**438.1**
1,4-Pregnadien-17,21-diol-3,20-dione	344.4	**345.2 (100)**		408.2 (27)	327.1 (17)	309.1 (18)		**120.9**
1,4-Pregnadien-6α-methyl-17,21-diol-3,11,20-trione	372.5	**373.1 (100)**	414.2 (16)	436.1 (77)	355.1 (15)			**436.1**
1,4-Pregnadien-l1β,21-diol-3,20-dione	344.4	345.2 (62)		408.2 (11)	**327.2 (100)**	309.1 (24)	291.1 (23)	**172.9**
1,4-Pregnadien-16β-methyl-17,21-diol-3,11,20-trione	372.5	**373.2 (100)**		436.3 (49)	355.2 (19)			**146.9**
Desonide	416.5	417.2 (99)		480.3 (58)	**399.2(100)**			**146.9**
Prednisolone acetate	402.5	403.2 (73)		466.2 (77)	**385.2 (100)**	367.2 (20)		**146.9**
1,4-Pregnadien-6α-methyl-11β,17-diol-3,20-dione	358.5	**359.2 (63)**		422.3 (74)	**341.2 (100)**	323.2 (51)	305.1 (14)	**422.3**
1,4-Pregnadien-21-ol-3,20-dione	328.4	329.2 (100)		392.2 (31)	311.1 (15)	293.1 (24)		**120.9**
Budesonide R-epimer	430.5	431.3 (86)		494.3 (46)	**413.3 (100)**			**146.9**
Budesonide S-epimer	430.5	431.3 (93)		494.3 (47)	**413.3 (100)**			**146.9**

Numbers in parentheses represent the relative peak intensity with respect to the base peak. The base peaks at cone voltage = 50 V are also shown. Base peaks are shown in bold font.

Table 7
Major ions in mass spectra of Group III corticosteroids at cone voltage = 25 V

Steroids	MW	$[M+H]^+$	$[M+Na]^+$	$[M+K]^+$	$[M+H+CH_3CN]^+$	$[M+Na+CH_3CN]^+$	$[M+H-HF]^+$	$[M+H-2HF]^+$	$[M+H-HF-H_2O]^+$	$[M+H-HF-2H_2O]^+$	$[M+H-HF-3H_2O]^+$	Base peak at 50 V
Triamcinolone	394.4	395.2 (56)	417.3 (28)			458.3 (60)	**375.2 (100)**		357.2 (25)	339.2 (15)	321.1 (10)	**458.3**
Triamcinolone acetonide	434.5	435.3 (77)				498.3 (54)	**415.3 (100)**		397.3 (18)			**498.3**
Triamcinolone diacetate	478.5	479.1 (63)	**501.1 (100)**	517.0 (17)		542.1 (68)	459.1 (36)		441.1 (25)			**501.1**
Fluocinolone acetate	452.5	**453.2 (100)**			494.2 (62)	516.2 (63)	433.2 (38)	413.1 (15)				**453.2**

Numbers in parentheses represent the relative peak intensity with respect to the base peak. The base peaks at cone voltage = 50 V are also shown. Base peaks are shown in bold font.

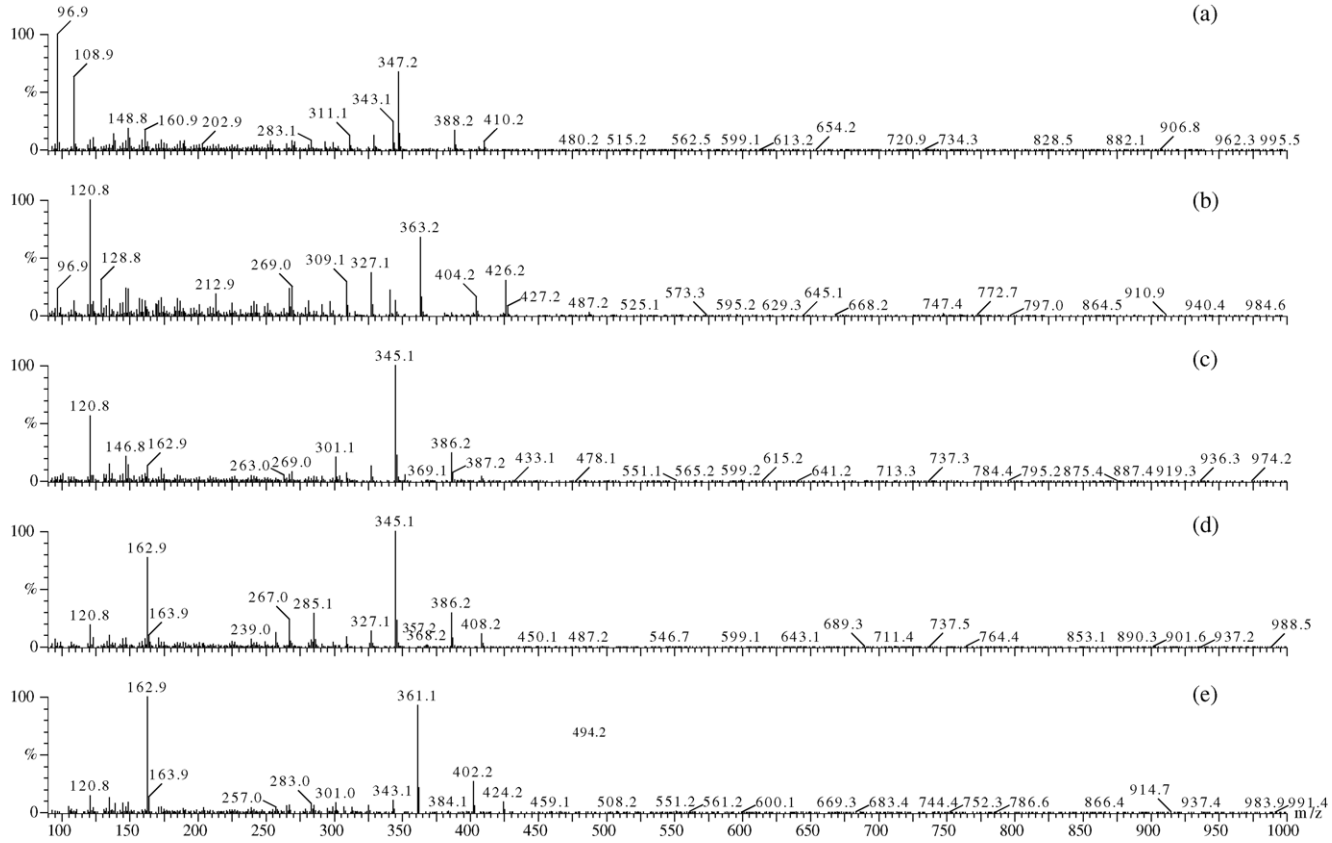
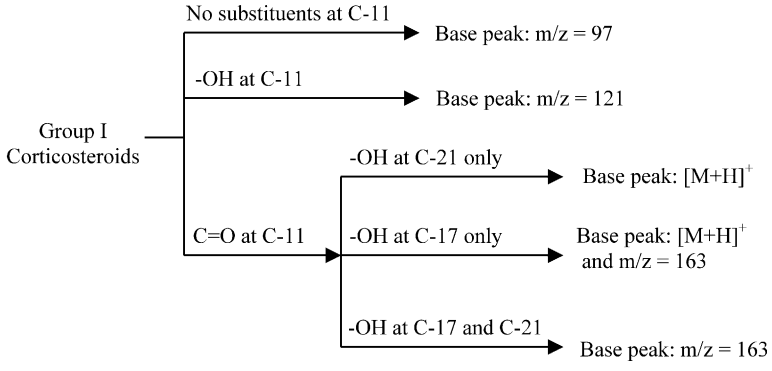


Fig. 4. Mass spectra of (a) Reichestein's substance S; (b) hydrocortisone; (c) 11-dehydrocorticosterone; (d) 21-deoxycortisone; and (e) cortisone obtained by LC–MS at cone voltage of 50 V.

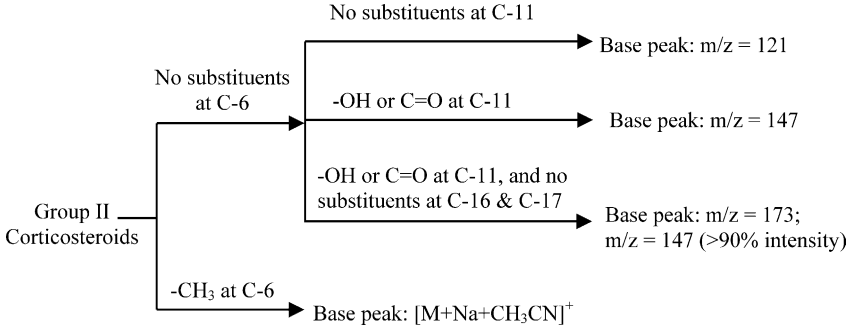
3.4.2. Group II, 1,4-pregnene-3-one corticosteroids without fluorine substituents

In contrast to Group I corticosteroids, the $[M+H]^+$ ion intensities were significantly reduced for Group II corticosteroids at a cone voltage of 50 V. The base peaks for these corticosteroids were mainly dependent on the substituents at C-11 and C-6, as shown in Scheme 2.

For corticosteroids without a methyl group at C-6, fragments $m/z = 121$, 147, and 173 were commonly observed in the mass spectra. When substituents were absent at C-11 (e.g., 1,4-pregnadien-17,21-diol-3,20-dione, and 1,4-pregnadien-21-ol-3,20-dione), fragment $m/z = 121$ formed the base peak. However, when C-11 contained a hydroxyl or carbonyl substituent the fragment $m/z = 147$ formed the base peak. 1,4-pregnadien-11β,21-diol-3,20-dione which had no substituents at both C-16 and C-17 was exceptional. This showed fragment $m/z = 173$ as the base peak, with fragment $m/z = 147$ present at about 90% relative intensity. Interestingly, when a methyl substituent was present at C-6, adducts of $[M + Na + CH_3CN]^+$ were observed as base peaks. These corticosteroids had fragments of $m/z = 135$, 161, and 187, which had mass differences = 14(CH_2) with



Scheme 1.



Scheme 2.

respect to $m/z = 121$, 147, and 173, suggesting that all fragments included the A-ring and probably the C-6 portion in their structures. This unique characteristic could be used to elucidate the presence or absence of $-CH_3$ at C-6.

3.4.3. Group III, 1,4-pregnene-3-one corticosteroids with fluorine substituents

The adduct $[M + Na + CH_3CN]^+$ was observed as the base peak for triamcinolone, triamcinolone acetonide and fluocinolone acetonide. The $[M + H]^+$ ions were observed at relatively low intensities (5–19% with respect to the base peak). Fragments $m/z = 121$ and 147 were also produced and their relative intensities were structure-dependent. Similar to the cone voltage = 25 V situation, the adduct $[M + Na]^+$ formed the base peak for triamcinolone diacetate. All fragments were produced with very low relative intensities (<20%) for triamcinolone diacetate, suggesting that the formation of acetate at C-16 and C-21 favored the formation of the $[M + Na]^+$ and $[M + Na + CH_3CN]^+$ adducts.

3.5. Fragmentation pathways of corticosteroids

Thalen [26] has reported that fragmentation ions of steroids can be divided into two classes: (I) fragments with the fused ring nucleus intact and (II) fragments formed from ring cleavage. In our studies, both classes were observed. All dehydration fragments, such as $[M + H - H_2O]^+$ and $[M + H - 2H_2O]^+$, belonged to Thalen's Class I fragments. These also included the fission of the C-17 side chains. For example, for budesonide (Fig. 3) and desonide, $m/z = 341$ and 323 were produced due to the loss of the acetal side chain at C-16 and C-17 and dehydration, and the further loss of the C-17 hydroxyacetone side chain ($-COCH_2OH = 59$ Da) to produce a fragment of $m/z = 265$. Fragments formed due to the cleavage of the C-17 hydroxyacetone side chain plus the loss of a water molecule were also observed. In our studies, Class I fragmentation was observed at both cone voltages of 25 and 50 V, which gave important information related to the molecular weights of the corticosteroids.

The low mass fragments, such as $m/z = 97$, 121, 147, 163, and 173, resulted from Thalen's Class II ring fragmentation [26]. Cleavages mainly occurred in the B- and C-rings, to produce fragments composing of the A-ring [26–28]. The presence of such low mass fragments in the spectra is evidence of the structural integrity of the A-ring [27]. Cleavage pathway (b) in Fig. 1 produced a fragment $m/z = 121$, the mechanism of formation of this fragment has been previously discussed [26,27]. The fragment $m/z = 135$, was produced by cleavage pathway (c) in Fig. 1. Fragment $m/z = 147$ is believed to result from cleavage along pathway (d) in Fig. 1, including the cleavage of the B-ring and C-ring [26]. Thalen observed that the formation of these fragments ($m/z = 121$, 135, and 147) was not affected by the presence of fluorine atoms at C-9 [26]. In our studies, the Group III corticosteroids had fluorine(s) at C-9 or at both C-9 and C-6. Although these fragments were not observed as base peaks, they were the predominant fragments in this low molecular mass region. Fragment $m/z = 163$ was produced from the cleavage pathway (e) in Fig. 1. Grostic and Rinehart observed that $m/z = 163$ was one of the major fragments for some 4-pregnene-3-one steroids [28]. They proposed that the formation of $m/z = 163$ was due to the cleavage of C-ring, accompanying with the fission of the C-9 and C-11 bond. Pathway (f) in Fig. 1, combining the cleavage of C-ring and the loss of a water molecule, was proposed to be responsible for the formation of $m/z = 173$ [26–27]. Cleavage pathway (a) in Fig. 1, along with some hydrogen transfers, probably led to the formation of fragment $m/z = 97$, however there was no literature available to support this hypothesis. Thalen proposed that these low mass fragments could be used to classify the 4-pregnene-3-one and 1,4-pregnadien-3-one steroids, as well as identify the presence/absence of substituents at C-6 [26]. In our studies, 4-pregnene-3-one corticorsteroids (Group I) appeared to form $m/z = 97$, 121, and 163, while the 1,4-pregnadien-3-one corticorsteroids (Groups II and III) mainly showed fragmentation to $m/z = 121$, 147, and 173. For Group II corticosteroids with methyl group ($-CH_3$) presented at C-6 position, such as methylprednisolone, fragments of $m/z = 135$, 161, and 187 were predominant fragmentation ions. All of these fragments had

mass difference of 14 ($-CH_2$) with respect to m/z = 121, 147, and 173.

4. Conclusions

The chromatographic and mass spectra properties of 23 corticosteroids were analyzed and related to their known chemical structures. These HPLC chromatographic and mass spectral characteristics of corticosteroids will be used for the elucidation and identification of unknown corticosteroids, degradation products and/or metabolites of known corticosteroids.

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