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The identification of related substances in triamcinolone acetonide by means of high-performance liquid chromatography with diode array detector and mass spectrometry

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Abstract: A study of an HPLC method for the analysis of related substances in triamcinolone acetonide is described.

Several systems of solvents and samples of different lots and preparative origins were examined and a rapid-scanning diode array UV detector (DAD) was particularly useful.

With the proposed technique it was possible to identify 9α-bromo desonide as a principal impurity, which was present in all examined samples of triamcinolone acetonide. This identification was rendered possible by the investigation of the second derivative of the UV spectra and by means of study of the mass spectrum. Furthermore, it was possible, primarily on the basis of the spectrophotometric data, to formulate reliable hypotheses on the possible identification of 9β, 11β-epoxide of the desonide which was present at very low levels and to exclude the presence of 11-deoxy-9(11)-unsaturated desonide. The presence of the above-mentioned related substances was explained considering the scheme of synthesis described in the literature. The spectrophotometric characteristics of the studied compounds and the limits of applicability of the present procedure are discussed.

Keywords: *Triamcinolone acetonide; HPLC; diode array detector; UV spectra, second derivative; mass spectrometry.*

Introduction

The necessity of developing an HPLC method for the identification and evaluation of related substances in triamcinolone acetonide samples, which could be proposed as a pharmacopoeial method, prompted us to undertake this study. Toward this goal several systems of solvents and samples of different lots and preparative origins were examined and the use of a rapid-scanning diode-array UV detector was found particularly useful for evaluating eluent systems. In fact as previously described [1], this detector allowed us not only to spectrophotometrically evaluate a compound revealed as a peak but also to verify its level of homogeneity.

Using appropriate reference compounds for comparison of the spectrophotometric properties, together with the auxiliary technology of mass spectrometry, we were able to identify the principal impurity in triamcinolone acetonide and to formulate a reliable hypothesis about the nature of the other related substances. These findings and the experimental protocol developed could prove very useful to researchers faced with similar investigations.

Experimental

Materials

Three samples of triamcinolone acetonide labelled 1L, 2FE and 3S were examined.

The liquid chromatographic solvents used were: acetonitrile for HPLC (Merck, Darmstadt, Germany), methanol for HPLC (Carlo Erba, Milan, Italy), chloroform for HPLC, stabilized with amylene (Carlo Erba) and water which had previously been purified by passage through a Millipore 'Milli-Q' apparatus. All mobile phases were filtered through 0.45 μm-pore filters.

Chromatographic procedures

Equipment. HPLC analyses were performed using:

(a) a liquid chromatograph composed of a programmer (Waters Automated Gradient Controller equipped with two Waters Pumps, model M-6000A and M-45), a Hewlett–

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Packard 1040A diode array detector (DAD) equipped with an HP-9000-300 computer, a colour monitor, and an HP-9153 disk drive, an HP Think Jet printer, an HP-Color Pro Plotter and a Rheodyne 7125 (20 μl loop) injection valve for reversed-phase chromatography;

(b) a liquid chromatograph composed of a Perkin–Elmer Series 410 Four-Solvent Pump System, a Perkin–Elmer LC-95 variable wavelength detector, a Hewlett–Packard 3390A recorder–integrator and a Rheodyne 7125 (20 μl loop) injection valve for normal-phase and preparative chromatography.

Mass spectra were obtained with a Hewlett–Packard HP-5988A mass spectrometer equipped with an HP 59970C MS ChemStation.

Chromatographic conditions. For reversed-phase chromatography a Spherisorb ODS 5 μm column (250 × 4.6 mm i.d.) was used. Two mobile phases for the isocratic elution were used: (a) methanol–water (65:35, v/v); and (b) acetonitrile–water (36:64, v/v).

All analyses were conducted at room temperature (25°C), the flow rate was 1 ml min^{-1} and the injection volume was 20 μl (fixed loop); the monitoring wavelength used with the DAD was 240 nm.

For normal-phase chromatography a Lichrosorb Si-100, 5 μm column (250 × 4.6 nm i.d.) was used. The mobile phase was: chloroform (stabilized with amylene)–methanol–water (978.6:20:1.4, v/v/v). All analyses were conducted at room temperature (25°C), the flow rate was 1 ml min^{-1}, the detection wavelength was 254 nm and the sensitivity was 0.2 A.U.F.S. The injection volume was 20 μl (fixed loop).

Preparation of sample solutions. Quantitative evaluation of related substances was performed using 50 μg samples and following the procedure described in one of our previous reports [2].

For the mass spectrometric identification of the impurity at r.r.t. 0.92 by normal-phase preparative chromatography (principal impurity which corresponds to the peak at r.r.t. 1.55 in the eluent (b), reversed-phase chromatography), a preparative chromatography of sample 1L was performed. Normal-phase chromatography was used because the impurity elutes before triamcinolone acetonide, allowing for minimal interference from residual traces of the principal peak. Three injections of 500 μg/20 μl of sample 1L, corresponding to three fractions of approximately 10 μg of impurity at r.r.t. 0.92 each, were performed.

For identification of related substances using reversed-phase HPLC methanolic solutions (1 mg ml^{-1}) were prepared of the following reference substances which corresponded to potential related substances: (1) 16α, 17,21-trihydroxypregn-1,4,9(11)trien-3,20-dione-16,17 acetonide (11-deoxy-9,11 unsaturated desonide); (2) 9α-bromo-11β, 16α, 17,21-tetrahydroxypregn-1,4-dien-3,20-dione-16,17-acetonide (9α-bromo desonide); (3) 9β,11β-epoxy-16α, 17,21-trihydroxypregn-1,4-dien-3,20-dione-16,17-acetonide (9β,11β-epoxy desonide).

After being appropriately diluted with methanol 1/20, these solutions were subsequently injected individually in order to study their chromatographic and spectrophotometric behaviour. To confirm their presence in sample 1L, 0.5 μg of each of these potential impurities were individually added to separate 50 μg/20 μl portions of the sample and injected.

Results and Discussion

Detection and quantitative evaluation of related substances

Normal-phase chromatography. Results of analyses of samples 1L, 2FE, 3S are summarized in Table 1. From our data follows that samples 1L and 2FE show similar profiles (Fig. 1) with only differences in total content (2.49 and 0.64%), both samples being characterized by a prominent impurity which is over 2% in sample 1L. Sample 3S shows a distinctly lower total impurity content (approx. 0.2%): on this sample only tentative identifications based on r.r.t. could be performed.

Reversed-phase chromatography. For the evaluation of impurities only mobile phase (b) was used since although similar profiles were obtained with both phases (a) and (b), better resolution was obtained with mobile phase (b) (Fig. 2). Table 1 summarizes the data. Results are in agreement with those obtained by normal-phase chromatography.

Identification of related substances

Sample 1L was selected for the attempt to

Table 1
HPLC retention data and quantitative evaluation for triamcinolone acetonide impurities

Normal-phase chromatography				Reversed-phase chromatography			
	Per cent content of samples				Per cent content of samples		
r.r.t.*	1L	2PE	3S	r.r.t.	1L	2PE	3S
0.27	—	—	traces	0.33	—	‡	traces
0.34	0.15	0.07	traces	0.88	†	‡	traces
0.53	—	—	traces	1.25	—	‡	traces
0.63	—	—	traces	1.34	†	‡	traces
0.92	2.28	0.57	traces	1.44	†	—	traces
1.13	0.06	traces	traces	1.55	1.99	0.48	traces
Total	2.49	0.64	0.2	1.85	—	‡	traces
Total of					†0.2	‡0.29	—
General total					2.2	0.77	0.2

* All r.r.t. (relative retention times) are referred to the retention time of triamcinolone acetonide.

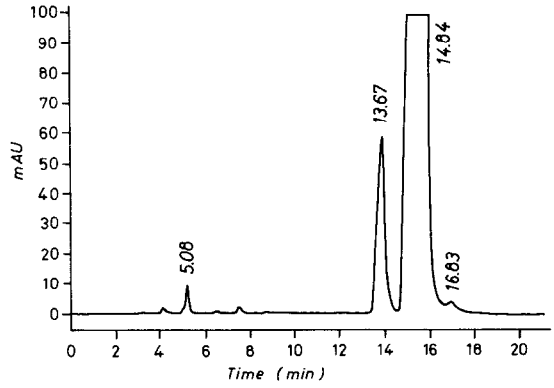

Figure 1
Normal-phase chromatography of sample 1L, 50 μg. Column: Lichrosorb Si-100, 5 μm, 250 × 4.6 mm i.d. Mobile phase: chloroform (stabilized with amylene)–methanol–water (978.6:20:1.4, v/v/v). Flow rate: 1 ml min^{-1}; detector wavelength: 254 nm; temperature: ≈25°C.

identify the impurities since it contained a higher content of related compounds.

Chromatography was performed using mobile phase (b) and the DAD. Comparisons were performed between sample and reference compounds by (1) direct r.r.t. measurements and (2) after adding small quantities of the presumed component to the sample. In addition, zero, first and second order spectra were compared [3].

Identification of 9α-bromo desonide. The principal impurity (r.r.t. 1.55, 1.99%) was observed to have the same chromatographic and spectrophotometric behaviour as a reference sample of 9α-bromo desonide. For this

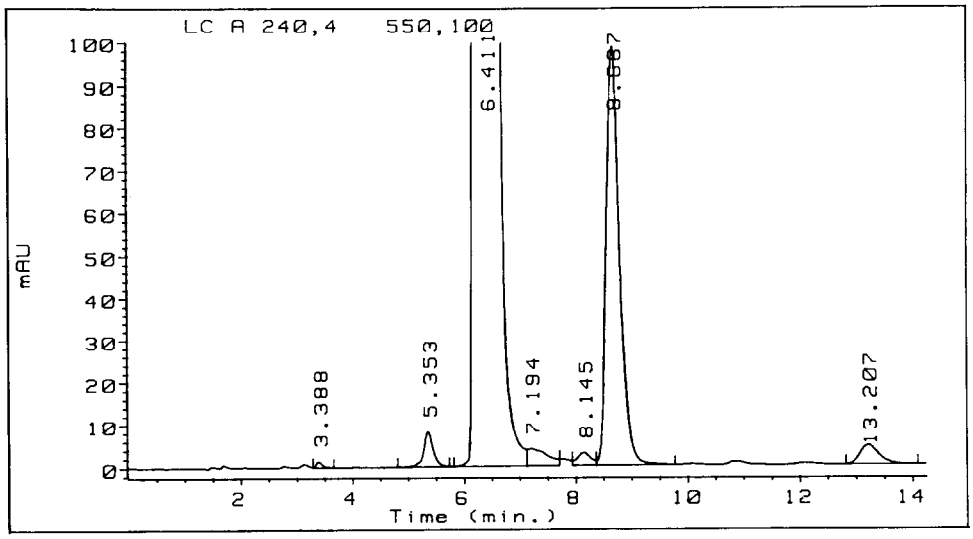

Figure 2
Reversed-phase chromatography of sample 1L, 50 μg. Column: Spherisorb ODS-2, 5 μm, 250 × 4.6 mm i.d. Mobile phase: acetonitrile–water (36:64, v/v). Flow rate: 1 ml min^{-1}; detector wavelength: 240 nm; temperature: ≈25°C.

Table 2
UV spectroscopic data for triamcinolone acetonide impurities

Compound	r.r.t.	Wavelength maximum in the UV spectrum	Wavelength minima in the second derivative of the UV spectrum	Remarks (second derivative profile)
Triamcinolone acetonide	1.00	241.5	241.5 269.5	Prednisolone type
9β,11β-epoxide of desonide	1.33	250.5	250.5 —	Hydrocortisone type
9α-bromo desonide	1.55	245.5	245.5 277.5	Prednisolone type
11-deoxy-9,11-unsaturated desonide	1.81	241.5	241.5 271.5	Prednisolone type

substance the spectrum of zero order showed an absorption maximum at 245.5 nm, an enlarged band at 270–275 nm and a second derivative of the spectrum with two points of minimum absorbance at 245.5 nm (principal) and 277.5 nm (secondary) (Table 2). This compound showed a 'prednisolone type' second derivative profile in accordance with a previously published classification [1]. While of analogous profile, the behaviour of the second derivative of the spectrum was clearly different from the corresponding derivative of triamcinolone acetonide whose spectrum of zero order showed a similar progression but with a maximum at 241.5 nm and a less pronounced inflexion at about 268 nm. The second derivative of the spectrum also presented a 'prednisolone type' profile with a principal minimum at 241.5 nm but a secondary minimum at 269.5 nm, allowing a clear differentiation between the two spectra (Fig. 3).

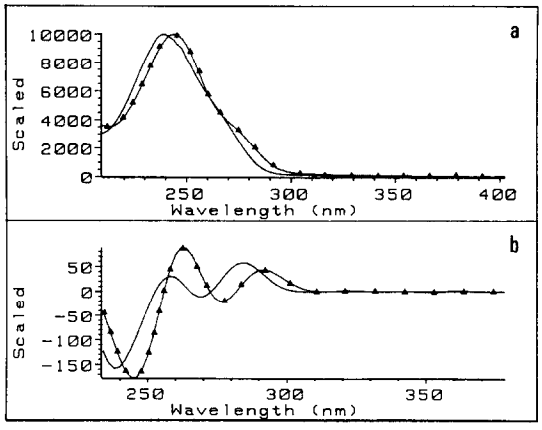

Figure 3
(a) UV spectrum of triamcinolone acetonide taken at the apex of the peak obtained by reversed-phase HPLC (see Fig. 2) (continuous line) and of the related substance at r.r.t. 1.55 (triangle line); (b) second derivatives of the UV spectra illustrated in (a).

The homogeneity (at over 90%) of the secondary peak at r.r.t. 1.55 (principal impurity) was verified by the comparison of the absorbance spectra taken at three points of the chromatographic peak: apex, leading and trailing edge. The spectra were found to be entirely superimposable (See ref. 1).

From a chromatographic point of view, the identity of the impurity was further confirmed by the coincidence of retention times between sample and reference compound obtained with direct-phase chromatography. The definitive confirmation of the structure of the impurity in question was obtained using mass spectrometry of the corresponding fraction isolated from preparative direct-phase chromatography as described later.

Verification of the presence of 11-deoxy-9,11-unsaturated desonide. The chromatographic and spectrophotometric behaviour of a sample of this substance was studied.

The resulting r.r.t. of 1.81 did not correspond to any of the impurities in sample 1L but was comparable to the r.r.t. of a minor impurity in sample 3S (r.r.t. 1.84).

The spectrophotometric data of this compound are reported in Table 2.

Verification of the presence of the 9β,11β-epoxide of the desonide. The chromatographic and spectrophotometric behaviour of a reference sample of this product was studied. Its r.r.t., at 1.33, corresponded to an impurity present in very minor quantities in all samples. The spectrum of zero order showed an absorption maximum at 250.5 nm, a second derivative with only one absorption minimum at 250.5 nm and showed a 'hydrocortisone type' profile [1] (see Fig. 4).

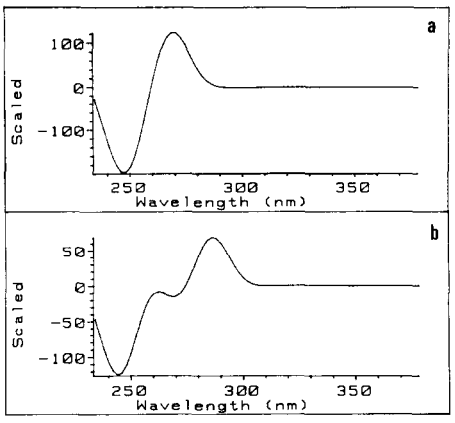

Figure 4
Profiles of second derivatives of UV spectra: (a) pregn-4en-3one (hydrocortisone type); (b) pregn-1,4-dien-3one (prednisolone type).

Isolation for mass spectrometry of the 9β,11-β-epoxide of the desonide in the sample 1L was impossible due to the extremely low amount of the corresponding peak present in all the samples. An attempt to obtain greater levels of impurity, in sufficient amounts to reach the detection limit by increasing the volume of the injected sample, was not effective due primarily to interference from the principal peak under these column loading conditions. However, the UV spectroscopic properties of the chromatographic peak were indicative of the presence of this kind of compound.

UV spectra of impurities

Our observations demonstrate how, by using second derivatives of the spectra, the differences between the zero order spectra of steroid compounds with a 4 en-3one chromophore and the parent compounds with a 1,4-dien-3one chromophore [4–6] can be enhanced (Fig. 4). The latter structure shows an enlarged band in the area of 263 nm which is due to the presence of a secondary maximum [Fig. 3(a)]. This maximum can be easily resolved with the use of the second derivative of the spectra and exactly identified by a second wavelength minimum. This criterion was shown to be particularly evident with compounds containing a halogenated substituent in position 9. Our observations confirm literature data on these compounds [7–12], showing how compounds containing the above chromophores present small variations in the position of the maximum of the principal band due to the presence of a halogenated substituent in 9. Bathochromic effects are shown with a bromine substituent (+3 nm) and with a 9β-11β-epoxy group (+5 nm) approximately, whereas hypsochromic effects are shown by a fluorine substituent (−3 nm) and a negligible effect is shown for the 9–11 en structure. In addition the second band in the 1,4 diene-3one chromophore is more differentiated from the principal band, as demonstrated by its wavelength minimum in the second derivative (see Table 2 and Fig. 3).

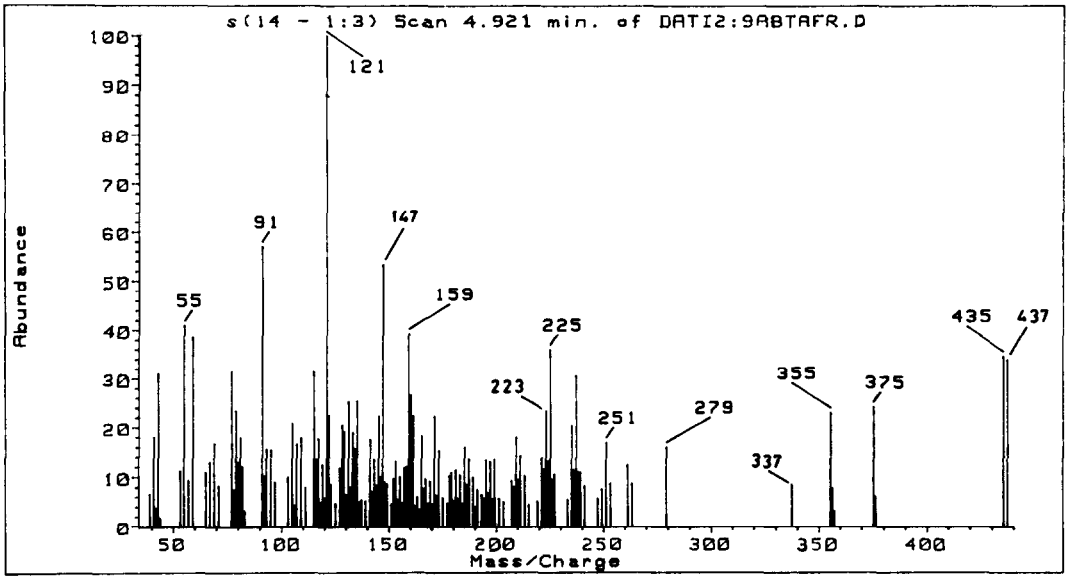

Figure 5
Mass spectrum of the fraction at r.r.t. 0.92 (principal impurity) isolated by HPLC (direct-phase chromatography) from sample 1L. Technique DIP, 70 eV.

The sensitivity of the proposed method for obtaining spectra from chromatographic peaks was evaluated by injecting into the column scalar quantities from 10 to 0.18 μg of 9β,11β-epoxide of the desonide. These quantities corresponded to absorbance values recorded at the maximum of the spectrum taken at the apex of the peak from ≈453 to ≈6.4 mAU. These results showed that with values of approximately 20 mAU, corresponding to a quantity of about 0.5 μg of a compound with this chromophore, it is possible to obtain absorbance spectra as well as first and second order derivatives. This provides a useful element of correlation with the number of double bonds in the A-ring and with the presence of some substituents in the B-ring.

Confirmation of the identifications of the 9α-bromo desonide by means of mass spectrometry

The mass spectrum of the impurity at r.r.t. 0.92 in the direct-phase chromatography was compared with those obtained from the synthesized 9α-bromo desonide. The impurity fraction was verified homogeneous by re-chromatography in reversed-phase with eluent (b) and DAD spectrophotometric analysis.

A complete MS of 9α-bromo desonide is not easy to obtain by means of the usual DIP (Direct Introduction Probe) by electronic impact (E.I.), at 70 eV, by heating from room temperature to 250°C at a velocity of 45°C min.

The spectrum of the sample isolated by normal-phase chromatography, shown in Fig. 5 was obtained on a TIC (total ion current) peak at 6.3 min. In this spectrum the following ions can be observed, from the higher to the lower masses: *m/z* 437 and *m/z* 435, two ions of approximately equal intensity (48.1%). The second ion can be attributed to the mass A = (M-59) or M-(CO—CH_2OH). These two ions confirm the presence of bromine in the molecule, based on the characteristic of monobrominated products to show an isotopic ion at M + 2 of an intensity practically equal to that of the ion M.

Further ions observed in this spectrum were: *m/z* 414 (from M-HBr); *m/z* 355 (from mass A-HBr = mass B); *m/z* 337 (from mass B-H_2O = mass C); *m/z* 279 (from mass 337-CH_3COCH_3: confirmation of an acetonide structure); *m/z* 223 and 225 (opening of the D ring); *m/z* 121 (opening of the B ring with loss of the entire C ring).

In this spectrum the molecular ion at *m/z* 494 was not observed. An extra ion at *m/z* 375 was present in low amounts in this spectrum but not in that of the synthesized product, obtained under the same conditions, otherwise the two spectra corresponded with each other (Figs 5 and 6). The extra ion might be explained by the presence of traces of triamcinolone acetonide, the principal product from which the impurity was isolated. In fact, the mass spectrum of

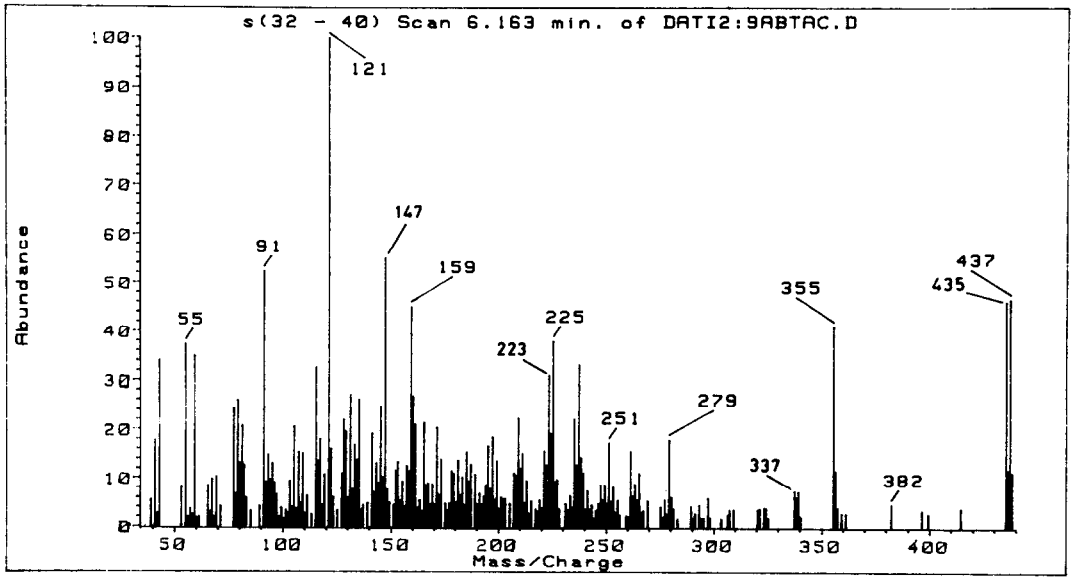

Figure 6
Mass spectrum of 9α-bromo desonide, authentic sample. Technique DIP, 70 eV; peak at 6.3 min of TIC (total ion current).

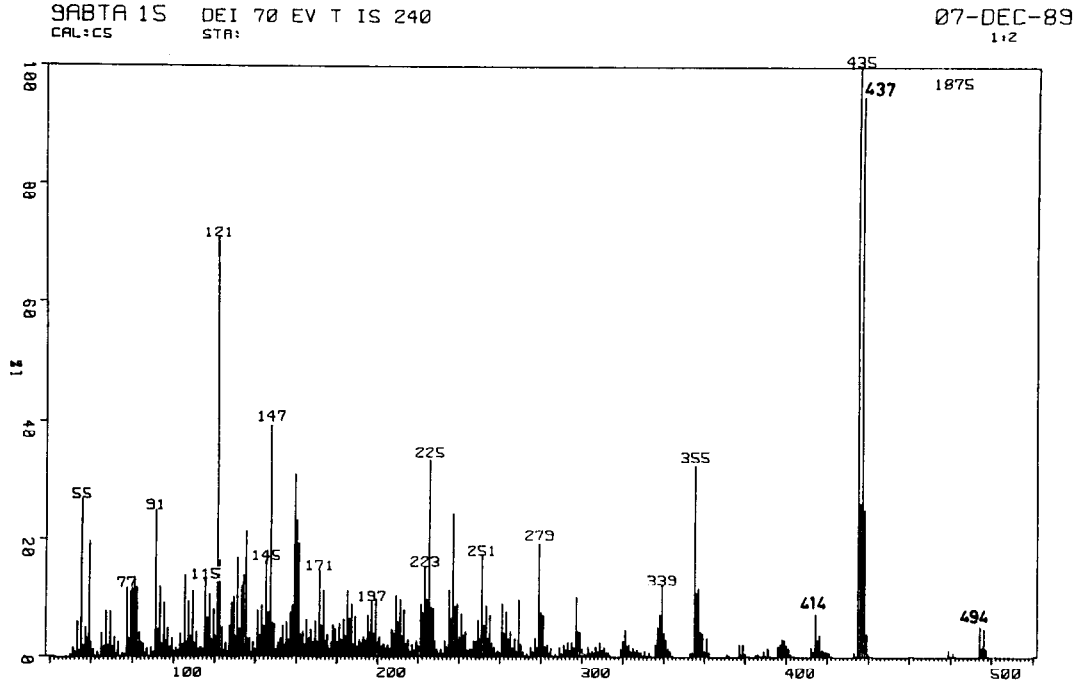

Figure 7
Mass spectrum of 9α-bromo desonide, authentic sample. Technique DEI, 70 eV.

triamcinolone acetonide, showed ions at 434, 375 (95, 3% abundance), 317, 279, 237, 159, 147, 121, 91, corresponding to previously published data [13, 14].

The mass spectrum obtained on a second peak of TIC at 4.3 min (not shown here) was characterized by a thermal elimination of HBr: in fact the more elevated mass is at *m/z* 414 (494-80).

By using a different ionization technique, i.e. the direct electronic impact ionization technique (DEI), it was possible to observe, while at low percentage abundance levels, the presence of the molecular ion at *m/z* 494, accompanied by that at *m/z* 496 in approximately equal quantities (Fig. 7).

Using this DEI technique, the mass at greater relative abundance was that at *m/z* 435 followed by that at *m/z* 437. The presence of the successive masses at *m/z* 414, 355, 339, 279, 223, 147 and 121 corresponded to the DIP spectra.

The mass spectrometric analysis of the small HPLC-isolated sample of impurity allows the attribution of the structure of 9α-bromo desonide to the product. This compound is an intermediate in the synthesis of triamcinolone acetonide.

Source of impurities

Synthetic processes currently used for the halogenation in the 9 position of 16-hydroxy prednisolone-16α,17α acetonide (desonide, prednacinolone) have been previously described [15–18].

The introduction of a halogen in 9 in the desonide structure is usually performed following the procedure described by Fried and by Sabo *et al.* [7, 8, 19]: in this procedure key intermediates can be 9(11) dehydro desonide (I), 9α-bromo desonide (II) and 9β, 11β epoxide of desonide III, and so these compounds are potential impurities.

Conclusion

This chromatographic and spectrophotometric approach may contribute to an initial diagnosis of the nature of the secondary peaks present in chromatograms of corticosteroids of which the purity must be evaluated: this assumption is confirmed in a separate publication [20] on related substances of 9α-fluoroprednisolone acetate.

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References

[1] G. Cavina, R. Alimenti, B. Gallinella, R. Porrà and L. Valvo, Atti del 4° Convegno Nazionale di Analitica Farmaceutica, Milano, 24 November 1988, p. B1–B11, Auditorium Farmitalia.

[2] G. Cavina, L. Valvo and R. Alimenti, *J. Pharm. Biomed. Anal.* **3**, 536–546 (1985).

[3] A.F. Fell, *Proc. Analyt. Div. Chem. Soc.* **15**, 260–267 (1978).

[4] H. Rössler and E. Brückner, *Naturwiss.* **48**, 695 (1968).

[5] E. Ivashkiv, *Biotechn. Bioeng.* **8**, 561–567 (1971).

[6] European Pharmacopoeia, Maisonneuve S.A., vol. II, p. 165, "Betamethasone", 1st edn (1971).

[7] J. Fried, K. Florey, E.F. Sabo, J.E. Herz, A.R. Restivo, A. Borman and F.M. Singer, *J. Am. Chem. Soc.* **77**, 4181–4182 (1955).

[8] J. Fried and E.F. Sabo, *J. Am. Chem. Soc.* **79**, 1130–1141 (1957).

[9] J.P. Dusza, M. Heller and S. Bernstein, in *Physical Properties of the Steroid Hormones* (L.L. Engel, Ed.), p. 95. Pergamon Press, Oxford (1963).

[10] G.R. Allen Jr and M.J. Weiss, *J. Amer. Chem. Soc.* **81**, 4968–4979 (1959).

[11] E.P. Oliveto, R. Rausser, L. Weber, A.L. Nussbaum, W. Gerbet, C.T. Coniglio, E.B. Hersberg, S. Tolksdorf, M. Eisler, P.I. Perlman and M.M. Pechet, *J. Am. Chem. Soc.* **80**, 4431 (1958).

[12] E.P. Oliveto, R. Rausser, H.L. Herzog, E.B. Hersberg, S. Tolksdorf, M. Eisler, P.L. Perlman and M.M. Pechet, *J. Am. Chem. Soc.* **80**, 6687–6688 (1958).

[13] K. Florey, *Analytical Profiles of Drug Substances*, Vol. 1, p. 397 (1972); Vol. 11, p. 615 (1982). Academic Press. New York.

[14] B.A. Lodge and P. Toft, *J. Pharm. Pharmacol.* **23**, 196–199 (1971).

[15] R.W. Thoma, J. Fried, S. Bonanno and P. Grabowich, *J. Am. Chem. Soc.* **79**, 4818 (1957).

[16] S. Bernstein, R.H. Lenhard, W.S. Allen, M. Heller, R. Littell, S.M. Stolar, L.I. Feldman and R.H. Blank, *J. Am. Chem. Soc.* **81**, 1689–1696 (1959).

[17] L. Mantica, R. Ciceri, J.P. Cassagne and E. Mascitelli Coriandoli, *Arzneim. Forsch.* **20**, 109–110 (1970).

[18] S. Bernstein, R. Littell, J.J. Brown and I. Ringold, *J. Amer. Chem. Soc.* **81**, 4573–4574 (1959).

[19] J. Fried and E.F. Sabo, *J. Am. Chem. Soc.* **75**, 2273–2274 (1953).

[20] G. Cavina, L. Valvo, B. Gallinella, R. Porrà and A.L. Savella, *J. Pharm. Biomed. Anal.* **10**, 437–446.

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