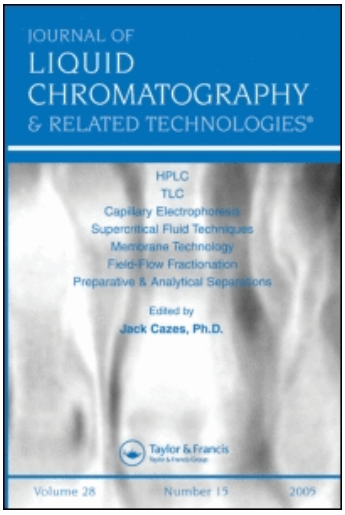

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HPLC-METHODS FOR SEPARATION AND QUANTITATION OF RESERPIN AND ITS MAIN DEGRADATION PRODUCTS

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ABSTRACT

An analytical method suitable for the stability control of dosage forms containing reserpine by HPLC is described. Besides reserpine the method quantitatively determines isoreserpine, 3,4-didehydroreserpine, trimethoxybenzoic acid, and renoxidine. The additional degradation products reserpic acid, and the secondary oxidation product 3,4,5,6-tetradehydroreserpine are qualitatively recorded.

INTRODUCTION

The chromatographic isolation and quantitative determination of reserpine by HPLC has been described by several authors. The emphasis has been put either on the separation of reserpine from accompanying alkaloids (1), metabolites (2), additional medicinal compounds (3), or degradation products in solid dosage forms (3, 4).

The method presented in addition allows to isolate and to quantify the main degradation products of reserpine formed by hydrolysis, isomerization, and by oxidation. It has been developed to investigate the kinetics of degradation of reserpine in solid dispersion with

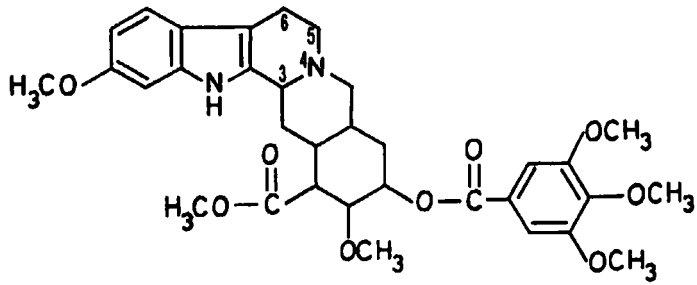

FIGURE 1: Molecular Structure of Reserpine

polyvinylpyrrolidone, Eudragit^{R} E (a combination of poly(butylmethacrylate), poly(2-dimethyl-aminomethyl methacrylate, and poly(methylmethacrylate)) respectively, and lactose (5, 6).

MATERIALS

Reserpine (Figure 1)

The material (batch 23, Guilini Pharma, D- Hannover) was kindly provided by Beiersdorf AG, D- Hamburg.

It was considered as pure after HPTLC and HPLC (degradation less than 0.1 %) and was used as received.

Degradation Products of Reserpine

Products of Oxidation

3,4-Didehydroreserpine (DDHR)

Ciba- Geigy, CH- Basel, kindly provided material (batch no. Sch-845-14G) for the purpose of comparison. Methods for the preparation and properties of this compound have been published (7, 8).

3,4,5,6-Tetradehydroreserpine (TDHR)

The material was prepared by a method based on that described by Wright and Tang (7), starting with 0.5 g reserpine. The main product of the reaction corresponded to data in the literature (9, 10) by qualitative HPTLC and by the maxima for excitation and emission in fluorescence spectroscopy. The blue fluorescence (excitation at 366 nm) differentiates this product from all other known products of degradation of reserpine. Therefore it is assumed that this compound is TDHR.

For the use as an external standard in HPLC, the product of the reaction was purified by LC first and then by HPTLC, using plates and eluents as decribed in (7). The zone containing the purified compound was then separated and eluated with methanol. The solution obtained was directly used for identification of unknown peaks in HPTLC and in HPLC. Crystalline material was not obtained.

Renoxidine (reserpine N-oxide)

In general, N-oxides may be prepared by oxidation of the parent amines by hydrogen peroxide or by peracids. Preliminary experiments revealed hydrogen peroxide as not selective. However, m-chloro perbenzoic acid was used in the N-oxidation of a series of alkaloids (11) and was applied to reserpine by Courts and Timmins (12). Therefore, renoxidine was prepared by the latter method, starting with 2.5 g reserpine.

The UV spectra and the fluorescence spectra of the product obtained were identical with those for reserpine, as was expected. The deviations between the IR spectra of reserpine and of renoxidine, first reported by Ulshafer et al. (13), were confirmed.

The molar absorptivity in methanol (15600 with 95 % confidence limits of ± 100) was lower by 6 % than that reported (13), while the determined and reported absorptivities for reserpine were identical. The deviation is attributed to byproducts. The lower melting point (221 °C accompanied by degradation, obtained by the method of Linström at 1 K min^{-1} and not corrected, as compared to a literature value of 238 to 241 °C) points in the same direction. HPTLC and LC did not reveal the nature of such by-products.

Products of Hydrolysis and of Isomerization

Ciba- Geigy, CH- Basel, kindly provided a sample of reserpic acid (batch C-27777-Su) for the purpose of identification.

Trimethoxybenzoic acid (TMBA) was obtained form Riedel de Haen, D- Hannover (prod. no. 63132) and used as received.

The product of isomerization, isoreserpine, was kindly provided in small quantity (batch Sch-930-5c) by Ciba-Geigy, CH- Basel, for the purpose of identification.

Eluents and Salts for HPLC

Water was prepared by destillation, other solvents were of special HPLC quality (Lichrosolv^{R}, E. Merck, D- Darmstadt), and salts were of analytical quality (p.a., E. Merck). Prior to use, the eluents were degassed by ultrasonic treatment.

Configuration of Analytical Equipment

1. HPLC dual piston pump with pulse dampener
   (Spectroflow 400, Kratos GmbH, D- Karlsruhe)
2. Sampling valve with 20 µl loop
   (RH 7125, Rheodyne Inc., USA- Cotati (CA))
3. HPLC column 250 * 4 mm, with guard column 30 * 4 mm
   (Vertex, Knauer GmbH, D- Berlin)
4. Separation phases: Nucleosil 5 C8
   (Macherey & Nagel GmbH & Co KG, D- Düren)
5. Spectrofluorometer with 2 monochromators and flow-through cell of 20 µl volume
   (SFM 23 B, Kontron GmbH, D- Eching b. München)
6. Integrator/ plotter
   (SP 4100, Spectra-Physics GmbH, D- Darmstadt)

METHODS

Sample Preparation

Reserpine and its products of degradation were dissolved in a mixture of acetonitrile and an aqueous solution of 0.5 % NH_4Cl 3+7 (v+v).

Quantitation

The peak areas were used according to the method of external standards. For each compound to be analyzed a separate standard was employed.

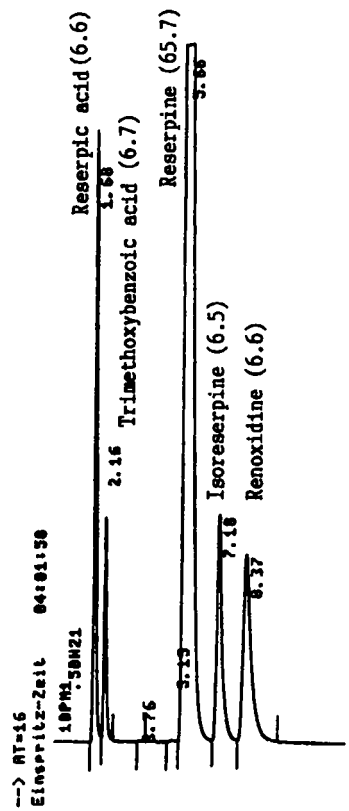

FIGURE 2: Chromatogram of standards in mixture (reserpine and degradation products according to Table 1 with retention times; eluent: acetonitrile and 0.5 % NH_4Cl in water 1 + 1 (v + v); numbers behind names indicate concentration in nmol·ml^{-1}; detection by fluorometry, excitation at 270 nm, emission at 360 nm)

RESULTS AND DISCUSSION

Isolation and Quantitation of Reserpine and its Products of Degradation

The liquid-chromatographic separation of reserpine on reverse phases by aqueous-organic eluents without salts causes very long retention times. As reported for many other alkaloids, these times may be shortened considerably by the addition of electrolytes (14).

TABLE 1
Standards and concentrations for chromatography to demonstrate selectivity by fluorometric analysis using excitation at 270 nm and emission at 360 nm.

Compound	Concentration	
	[$\mu g \cdot ml^{-1}$]	[$nmol \cdot ml^{-1}$]
Reserpine	40.0	65.7
3,5 Didehydroreserpine	4.25	7.0
Isoreserpine	3.99	6.5
Renoxidine	4.13	6.6
Reserpic acid	2.63	6.6
Trimethoxybenzoic acid	1.43	6.7

For a selective determination of reserpine and its products of degradation TMBA, isoreserpine, and renoxidine a mixture of equal volume parts of acetonitrile and an aqueous solution of 0.5 % NH_4Cl was suited best. At a flow rate of 1.5 $ml \cdot min^{-1}$ a pressure of about 25 MPa resulted. The wavelengths of excitation as well as of emission were set for the compound with the smallest intensity of fluorescence, TMBA (270 and 360 nm, respectively).

A sample chromatogram obtained for a mixture of standard compounds is depicted in Figure 2. Selectivity is demonstrated. All the compounds are separated completely. Especially in fluorometry, pharmaceutical excipients common in solid dosage forms do not interfere.

The concentrations for the compounds in Figure 2 are given in Table 1. The solvent was a mixture of acetonitrile and an aqueous solution of 0.5 % NH_4Cl 3+7 (v+v). Since the products of degradation DDHR and TDHR do not fluoresce at this set of wavelengths, they are not detected, as is shown in Figure 2. The quantitation of these latter compounds is described in the following chapter.

In order to assure the linearity of the signal of the detector, for each of the compounds in Table 1 one sample was weighed exactly and 6 differently concentrated solutions were prepared from this sample. Of each solution, 20 µl were injected and the areas of the resulting peaks were determined by integration. The results of a linear regression of the peak areas on the concentrations are collected in Table 2.

TABLE 2

Linearity of the fluorometric determination of reserpine and several degradation products by linear regression of peak areas (UA: units of area) on concentrations (b(0): intercept; b(1): slope; SD: standard deviation; r^2: coefficient of determination; c_{min} to c_{max}: range of concentration used)

	c_{min} [ng·20µl^{-1}]	cmax [ng·20µl^{-1}]	b(0) ± SD [UA]	b(1) ± SD [UA·20µl·ng^{-1}]	r^2
Reserpine	110	1200	2554 ±66962	2376 ± 85	0.9936
3,4-Didehydroreserpine	3	54	2395 ± 2192	17829 ± 85	1.000
Isoreserpine	2	25	-873 ± 412	2047 ± 26	0.999
Renoxidine	110	1100	3816 ±33504	1940 ± 46	0.9970
Reserpic acid	2	25	1663 ± 2816	8460 ± 175	0.996
Trimethoxybenzoic acid	1	10	-209 ±173	1109 ± 28	0.994

Selective Determination of 3,4-Didehydroreserpine and 3,4,5,6-Tetradehydroreserpine

The maxima for excitation and emission in fluorometry for DDHR and TDHR occur at higher wavelengths than for reserpine. Simultaneous detection was therefore not possible. On the other hand, reserpine and the other products of primary degradation do not interfere in the quantitation of DDHR and TDHR. Therefore, their determination would be possible without prior chromatographic separation, e.g. in a solution prepared directly from the sample. However, secondary products of continuing degradation may indicate too high concentrations of these compounds when such a non-selective method would be used. Therefore, DDHR and TDHR were quantitated as well after chromatographic separation by HPLC. A complete separation was not reached. This is not mandatory, if two consecutive, separate measurements are carried out, each at the specific maximum of fluorescence. These maxima differ enough to allow a selective determination.

TABLE 3

Retention times in [min] for reserpine and its main degradation products

Eluent 1: Methanol - 0,5 % NH_4Cl in Water (8 + 2)

Eluent 2: Acetonitrile - 0,5 % NH_4Cl in Water (1 + 1)

Compound	retention time [min]	
	eluent 1	eluent 2
Reserpic acid	2.84	1.68
Trimethoxybenzic acid	2.84	2.16
Reserpine	3.51	5.66
Isoreserpine	4.10	7.18
Renoxidine	4.64	8.37
3,4-Didehydroreserpine	4.39	8.60
3,4,5,6-Tetradehydroreserpine	4.84	8.68

The eluent mentioned in Figure 2 leads to retention times of as high as 8.60 min for DDHR and 8.68 min for TDHR. Another eluent was used in this case, in order to achieve narrower and higher peaks, to lower the limit of detection, and to raise the accuracy of the method: a mixture of methanol and an aqueous solution of 0.5 % NH_4Cl 8+2 (v+v).

Table 3 shows the retention times for reserpine and its products of degradation for this latter eluent (eluent 1) as compared to the mixture using acetonitrile instead of methanol, which was described in Figure 2 (eluent 2). The flow rates and pressures were 1 $ml \cdot min^{-1}$ and 25.5 MPa for eluent 1, and 1.5 $ml \cdot min^{-1}$ and 28.0 MPa for eluent 2.

The compounds no. 1 to 5 in Table 3 were determined using excitation at 270 nm and emission at 360 nm. The compound no. 6, DDHR, was determined using excitation at 390 nm and emission at 490 nm, compound TDHR at 340 and 440 nm respectively. It can be seen from Table 3 that eluent 1 results in better separation of DDHR from TDHR than eluent 2.

TABLE 4

Eluents for HPLC in assuring identity of unknown substances by co-chromatography

Eluent	flow rate [ml·min^{-1}]	pressure [MPa]
1 Methanol - 0,5 % NH_4Cl / H_2O (2 + 1)	1.0	320
2 Tetrahydrofuran - 0,5 % NH_4Cl / H_2O (2 + 3)	0.8	325
3 Acetonitril - 0,02 N-NaH_2PO_4 / H_2O (85 + 15)	1.5	106
4 Methanol - 0,02 N-NaH_2PO_4 / H_2O (8 + 2)	1.0	250
5 Tetrahydrofuran - 0,02 N-NaH_2PO_4 / H_2O (1 + 1)	0.8	340

TABLE 5

Retention times for reserpine and its products of degradation obtained by using 5 different eluents (Table 4) on the same column.

Compound	retention time [min] for eluent no.				
	1	2	3	4	5
Reserpic acid	3.14	3.12	5.41	2.74	2.84
Trimethoxybenzoic acid	3.14	5.84	2.83	2.49	4.21
Reserpine	5.50	5.89	6.46	4.73	5.69
Isoreserpine	7.62	8.44	4.83	4.95	0.22
Renoxidine	9.65	6.35	7.75	4.74	5.20
3,4-Didehydroreserpine	8.75	5.20	8.97	7.74	5.08
3,4,5,6-Tetradehydroreserpine	10.82	5.08	9.61	9.18	5.04

Alternative Eluents for Special Purposes

A comparison of the retention times of an unknown sample and known standards helps in the identification of unknown constituents. In addition, standard compounds can be added to the sample. With concentrations in the same order of magnitude, even small differences in retention times of the two compounds cause overlapping peaks which expose two maxima. This effect can be detected more clearly when the separation with several eluents is investigated. A range of binary eluents developed for this purpose (Table 4) contained as salts either ammonium chloride, as before, or sodium dihydrogenphosphate. Ternary eluents did not lead to better selectivity.

Table 5 collects the results obtained with the different eluents of Table 4 in the HPLC separation of reserpine and its products of degradation.

From the results in Table 5 reserpine and its products of degradation may be differentiated into 3 groups:

Group 1 is made up by the organic acids reserpic acid and TMBA. Regularly they are eluated first. Reserpic acid may be eluated i) before TMBA (with the eluents containing tetrahydrofurane or with a mixture of equal volumes of acetonitrile and 0.5 % aqueous NH_4Cl), ii) at nearly the same time as TMBA (with the eluents containing methanol, i.e. 1 and 4), or iii) after TMBA (with the eluent mixture of acetonitrile and aqueous 0.02 N- NaH_2PO_4 , 85 + 15 (v + v)).

Group 2 includes reserpine, isoreserpine, and renoxidine. Isoreserpine is eluated after reserpine with the exception of eluent 3. The eluents containing tetrahydrofurane markedly delay the eluation. The rank order of reserpine, isoreserpine, and renoxidine is different with nearly all eluents. The order cited above results either with eluent 1 or with the mixture of equal volumes of acetonitrile and aqueous 0.5 % NH_4Cl. With eluent 2 isoreserpine appears as the last one, with eluent 3 as the first among these compounds. Eluent 4 does not lead to sufficient separation; with eluent 5 renoxidine appears even before reserpine.

Group 3 is made up by the two products of dehydration, DDHR and TDHR. No eluent results in complete separation. When the eluent contains tetrahydrofurane (eluents 2 and 5), both compounds appear before reserpine. With eluents containing acetonitrile or methanol, these are the last compounds appearing in the chromatogram. The best separation was achieved with the eluents containing methanol, i.e. eluents 1 and 4. Eluents with tetrahydrofurane are least effective in separation.

In quantitative analysis, all the eluents in Table 4 were inferior to those in Table 2. In parts of the respective chromatograms, the former separated the constituents insufficiently. However, they have the advantage to result in characteristic changes in the relative positions of the peaks. Thus they help to assign the various peaks to particular compounds.

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