Acta Poloniae Pharmaceutica – Drug Research, Vol. 58 No. 4 pp. 263–268, 2001

ISSN 0001-6837
Polish Pharmaceutical Society

DRUG TECHNOLOGY

THE METHOD OF DAUNORUBICIN PURIFICATION

WOJCIECH SŁAWIŃSKI[1], IRENA OSZCZAPOWICZ[1],
KATARZYNA CICHOCKA[1] and ANDRZEJ ZIMNIAK[2]

[1] Institute of Biotechnology and Antibiotics, 5 Starościńska Str., 02–516 Warsaw, Poland;
[2] Faculty of Pharmacy, Medical University of Warsaw, 1 Banacha Str., 02–097 Warsaw, Poland

Abstract: The economical method of daunorubicin purification was evaulated.

Keywords: daunorubicin, anthracycline antibiotics, purification.

Daunorubicin (daunomycin), applied in the form of hydrochloride, (Cerubidine, Daunoblastin, Daunorubicin R.P.) belongs to a broad spectrum of antitumor drugs, which for over two decades have been used extensively for a treatment of cancer patients, mainly with acute leukemia and solid tumors. Recently daunorubicin in liposomal from (DaumoXome) is used as the first line chemotherapeutic agent for advanced HIV associated Kaposi's sarcoma (1). Moreover, it is an intermediate in the synthesis of other anthracyclines, such as doxorubicin and epidoxorubicin.

In the Institute of Biotechnology and Antibiotics, where this antibiotic is produced, investigations on the method of daunorubicin purification are continuously carried out.

There are several known methods of purification of this antibiotic. According to one of them daunorubicin hydrochloride was purified in a two step procedure, where at first crystallization from the mixture of methyl alcohol and chloroform was carried out. The obtained product of about 90% purity after drying at 50°C, was stirred with *n*–butyl alcohol and then, after filtration and washing, was dried at 60°C. The yield of this process was about 50% (2). In the other methods the product was crystallized from a mixture of dioxane and water (3) or after dissolution in methyl alcohol was chromatographed on silica gel column, using for elution a mixture of chloroform, methyl alcohol and acetic acid in the ratio of 82:18:1 (4). According to the last two methods hydrochloride of daunorubicin was obtained with the yield of 62–70%.

In another procedure (5) this antibiotic was dissolved in water, methyl or ethyl alcohol and then precipitated by addition of *n*–butyl alcohol, acetone, diisopropyl ether or hexane. The product, obtained with the yield no more than 80%, should be dried about seven days in the temperature about 60°C. Only in these conditions the content of solvent was lower than 0.19%.

These known methods of purification of daunorubicin hydrochloride have several disadvantages such as:

– the yield does not exceed 80%,

– in some procedures – two step process of purification is necessary,

– a large volume of used solvents, *e.g.* according to patent application (5), for crystallization of 1 g daunorubicin hydrochloride 110 ml of mixture of methyl and *n*–butyl alcohol should be used,

– a long time of drying at 50–60°C. Such temperature can cause degradation of daunorubicin.

The aim of this work was an improvement of the technology, enabling to obtain this product of quality according to requirement of European Pharmacopoeia (6) and with the yield satisfactory from economical point of view.

According to this Pharmacopoeia, the content of daunorubicin hydrochloride, calculated as anhydrous and free from solvents substance, should be at least 95%, the content of water $\leq$ 3.0%, the content of solvent $\leq$ 1.5%, and the content of related substances – no more than 2.0%, among them the content of daunorubicinol hydrochloride $\leq$ 1.5%.

EXPERIMENTAL

Materials and methods

All solvents used were of pure grade (POCh or Fluka).

Method of obtaining of crude daunorubicin hydrochloride

Crude daunorubicin hydrochloride was isolated from fermentation broth by known method (3) *i.e.* by successive filtration, absorption–desorption procedure on Amberlite IRC–50, extraction with chloroform, concentration of extracts, acidification and finally crystallization from the mixture of chloroform and methanol. The product of 81.2% purity (assay for anhydrous and solvent free substance – 97.3%, assay for related substances – 2.2%, assay for water and residual chloroform – 2.6 and 14% respectively) was obtained with the yield of 57%.

Method of purification of crude daunorubicin hydrochloride

Crude daunorubicin hydrochloride of 81.2% purity (400 g) was dissolved in 4 l of methanol and filtered. The filtrate was concentrated under reduced pressure to oil residue and then aliphatic chlorinated hydrocarbon (4.0–4.7 l) was added. The obtained solution was stirred slowly for 15–20 hours at the temperature of 15–22°C. The formed crystals were filtered off, washed twice with a mixture of methanol – chlorinated aliphatic hydrocarbon (1:3), twice with *n*–hexane and dried under reduced pressure at the temperature below 35°C. Daunorubicin hydrochloride was obtained with the yield 82.3–86.2% (Table 2).

Method of isolation of pure daunorubicin hydrochloride from fermentation broth

Fermentation broth (900 l), containing 720 g of daunorubicin in the form of free base, was acidified to pH 1.4, stirred and filtered. The filtrate was treated with the 10% solution of sodium carbonate to pH 4.5 and then passed through a column containing Amberlite IRC in hydrogen from. Afterwards the column was washed with water and daunorubicin was eluted with a solution containing water, methanol and sodium chloride. The combined eluents (300 l) were concentrated to 15 l under reduced pressure and extracted at pH 7.5 with chloroform (3 × 10 l). To the chloroform extracts water was added (20 l), pH was adjusted to 3.5 and then the aqueous phase was extracted (10 × 10 l) again at pH 7.5 with chloroform (3).

The combined chloroform extracts were concentrated under reduced pressure at 30°C to the oil residue, which contained 531 g of daunorubicin in the form of free base. This residue was dissolved in 10 l of a mixture of ethanol with methylene chloride (1:4) and then pH was adjusted to 2.7 by the addition of 10% ethanol solution of hydrochloride. The mixture was kept for 20 hours at the temperature of 20±2°C and thus the formed crystals were filtered off, washed with the mixture of absolute ethanol with methylene chloride (1:4) and *n*–hexane and then dried under reduced pressure at the temperature below 35°C.

Daunorubicin hydrochloride (448 g) was obtained with the yield of 82.0% calculated as daunorubicin–free base contained in chloroform extract, and with the yield 60.5% calculated as the starting amount of daunorubicin–free base. Purity of the product was 95.3% calculated as anhydrous and solvent free substance; assay for related substances – 1.9%, assay for water – 1.8%, assay for solvent – 0.1%.

Method of analysis

High pressure liquid chromatography (HPLC)

Analysis condition:
column: ODS HYPERSIL 5 μm, 4.6 × 200 mm
mobile phase: linear gradient – laurylsulphate sodium (1.44 mg), orthophosphoric acid (1.15 g), water (500 ml), acetonitrile (450 ml) and methanol (50 ml),
flow rate: 2.2 ml/min
detection: UV 254 nm.

Gas chromatography (GC)

column – HP–1, 30 m × 0.53 mm × 5.0 μm (cross linked 50% methyl siloxane)
carrier gas: helium
detector: Flame Ionization Detector.
Content of water was determined with Mettler DL 18 Karl Fischer automatic titrator.
IR spectra were recorded on a Magma Nicolet IR 550, Nicolet spectrophotometer in KBr pellets.
The powder X–ray diffraction crystallographic measurements have been performed with a HZG–4 diffractometer, by use of the CuK_α filtered radiation; the 2θ range was 5–45°, the step scan increment was 0.04° of 2θ, and the count time was 5 sec/step. Value of interplanar spacings d (Å) and intensities of signals I (%) were compared.

RESULTS AND DISCUSSIONS

According to our investigations daunorubicin hydrochloride of high purity, containing only small amount of solvents, lower than ≤ 0.1%, can be obtained after initial purification of fermentation broth, by slow crystallization from the concentrated methanol solution, but with a low yield. It was noticed that after addition of chloroform to this solution a rapid crystallization took place, and pure antibiotic was produced with a high yield but with a high content of chloroform in the range 10.4–14.0% by weight. This solvent could not be removed from the product, even after several days of drying in vacuum at about 50°C.

That can be explained by the assumption that solvent molecules are captured in the crystal lattice of daunorubicin hydrochloride. Similar fact was already reported in the case of N–bromo–acetyl–daunomycin–solvate, where – accordingly to the X–ray data – solvent molecules can be found in the large cavities present in each unit cell (7).

In the search for economical method of purification of daunorubicin hydrochloride applicability of several chlorinated aliphatic hydrocarbons in the mixture with aliphatic alcohols in crystallization process have been examined. The resulting products have been analyzed by such methods as the HPLC, GC, IR, and for the estimation of the crystalline phase content powder X–ray diffraction was applied.

In this procedure pure, crystalline daunorubicin hydrochloride has been obtained with a high yield, however, in the most cases, the content of solvent in the final product was in the range of 4–18%. Only when ethyl chloride or methylene chloride or *trans* 1,2–dichloroethylene were used, after drying under reduced pressure at the temperature below 35°C, residual solvents were detected in amounts ≤ 0.1% (Table 1).

It was found that formation of the product containing large amount of solvent is characteristic only for daunorubicin hydrochloride. In the case of other anthracycline antibiotics, such as epidaunorubicin, doxorubicin and epidoxorubicin, the crystallization procedure performed in analogous conditions, as presented in Table 1, gave in each case crystalline pure product, practically free of the solvent (≤0.1%). These results indicate that a possibility of formation of the products, containing considerable or negligible amounts of chlorinated aliphatic hydrocarbons, is strictly related to the structure of anthracycline antibiotics, particularly to the presence of OH group on the C–14 atom (doxorubicin and epidoxorubicin) as well as to the orientation of this group on the C–4' atom in daunosamine moiety (epidaunorubicin and epidoxorubicin).

Basing on the above results, the optimal conditions for purification of daunorubicin hydrochloride have been elaborated. Crude hydrochloride was dissolved in methyl or ethyl alcohol or in their mixtures, the obtained solution was filtered and then chlorinated aliphatic hydrocarbon such as ethyl chloride, methylene chloride, *trans* 1,2–dichloroethylene or their mixtures was added. After some minutes crystallization started and the obtained suspension was stirred for some hours. Aliphatic alcohol or mixture of the presented above alcohols was used in the range

Table 1. Crystalization of daunorubicin hydrochloride from the mixture of methanol and chlorinated aliphatic hydrocarbons

No.	Solvent used for crystalization	Solvent residue (GC) [%]	*Purity (HPLC) [%]
1	methylene chloride	0.02	99.5
2	chloroform	10.4	99.6
3	carbon tetrachloride	13.6	99.7
4	methanol	0.05	98.9
5	1,1,2,2-tetrachloroethane	18.0	99.5
6	tetrachloroethylene	15.1	99.5
7	1,1,1,2-tetrachloroethane	17.9	96.8
8	1,2 dichloroethane	7.0	99.5
9	trichloroethylene	16.3	99.6
10	*trans*-1,2-dichloroethylene	<0.02	98.6
11	ethyl chloride	<0.02	98.0

* purity calculated as anhydrous and solvent-free substance

Table 2. Crystallization of daunorubicin hydrochloride from the mixture of methanol and some chlorinated aliphatic hydrocarbons

Solvent used for crystallization	methylene chloride	methylene chloride	*trans*-1,2 dichloroethylene	ethyl chloride
Assay of daunorubicin hydrochloride $C_{27}H_{29}NO_{10} \cdot HCl$ calculated as anhydrous and solvent-free substance	98.1%	98.4%	96.5%	96.2%
Solvent residue	0.1%	0.05%	< 0.1%	< 0.1%
Assay of related substances: daunorubicinol hydrochloride daunorubicin aglicone	1.5% 0.7% 0.4%	1.4% 0.6% 0.35%	1.7% 1.0% 0.5%	1.6% 0.8% 0.5%
Water content (K.F. method)	1.7%	2.6%	1.7%	1.8%
Yield of the crystallization	85.0%	86.25%	82.3%	83.2%

Table 3. Values of interplanar spacing (d. in Å) and intensities of signals (I. in %) for crystalline daunorubicin hydrochloride

Product from chloroform		Product from 1,1,2,2-tetrachloroethane		Product from *trans* 1,2-dichloroethylene		Product from ethyl chloride	
D	I	d	I	d	I	d	I
17.28	32	17.06	78	14.19	49	14.95	29
12.15	45	14.60	41	11.14	100	14.27	40
10.98	20	12.45	78	8.97	66	11.16	100
7.69	50	12.21	100	7.60	95	10.00	29
6.86	30	10.85	58	7.13	38	8.97	58
6.05	32	10.62	48	6.63	73	7.58	84
5.88	100	7.74	90	5.98	44	7.42	29
5.53	75	6.92	42	5.58	83	7.14	34
5.22	22	6.15	48	5.01	66	6.64	66
4.89	55	6.05	61	4.59	64	5.97	35
4.77	46	5.96	97	4.48	79	5.57	64
4.60	50	5.04	65	4.22	75	5.51	47
4.51	29	4.83	79	4.14	62	5.02	50
4.22	29	4.61	76	3.80	84	4.60	47
4.15	21	4.56	87	3.64	48	4.50	54
4.09	20	4.43	66	3.50	39	4.29	47
4.03	26	4.05	77	3.19	33	4.23	57
3.83	28	3.88	72	2.531	23	4.13	48
3.64	21	3.68	67			3.80	57
		3.61	44			3.64	35
		3.50	67			3.61	36
		3.40	43			3.50	32
		3.22	47			3.40	29
		3.14	49			3.19	28
		2.463	31			2.922	22
						2.864	21
						2.529	16
						2.417	16
						2.240	15

from 5 to 35 ml, and chlorinated aliphatic hydrocarbon – in the range from 5 to 25 ml, with respect to 1 g of daunorubicin hydrochloride. The process of crystallization was carried out during no more than 20 hours at the temperature of about 20°C and a product after filtration and washing was dried at the temperature below 35°C.

According to this method daunorubicin hydrochloride was obtained with high yield in the range 82.3–86.2%, and purity which complied with the European Pharmacopoeia requirements (6). The received results were presented in Table 2.

In the IR spectra (KBr), differences between hydrochloride of daunorubicin obtained in the presence of methylene chloride, methyl chloride or *trans* 1,2–dichloroethylene and in the presence of others chlorinated aliphatic hydrocarbons were observed, particularly in the range 2000–3500 cm^{-1} characteristic for C–H, N–H, and O–H stretching vibrations. In this range for the first group of the above mentioned solvents, in the IR spectra of this antibiotic there were five bands at 2890, 2940, 2985, 3180, 3440 cm^{-1}, whereas for product from the second group only one broad band (3320–3380 cm^{-1}) respectively was observed. It can be due to different possibility of hydrogen bonds formation between daunorubicin hydrochloride and solvents used for crystallization.

To the estimation of crystalline phase content in the resulted samples of daunorubicin hydrochloride X–ray powder diffraction analysis was applied. The products obtained in the purification procedure by use of different solvents and containing large amounts of these solvents showed different diffraction patterns (Figure 1, Table 3). In contrast, daunorubicin hydrochloride, crystallized from *trans* 1,2–dichloroethylene or ethyl chloride, formed products practically with no content of organic solvents, was obtained as only one polymorphic form and their diffractions patterns were identical (Figure 1, Table 3).

Interplanar spacing and diffraction patterns for

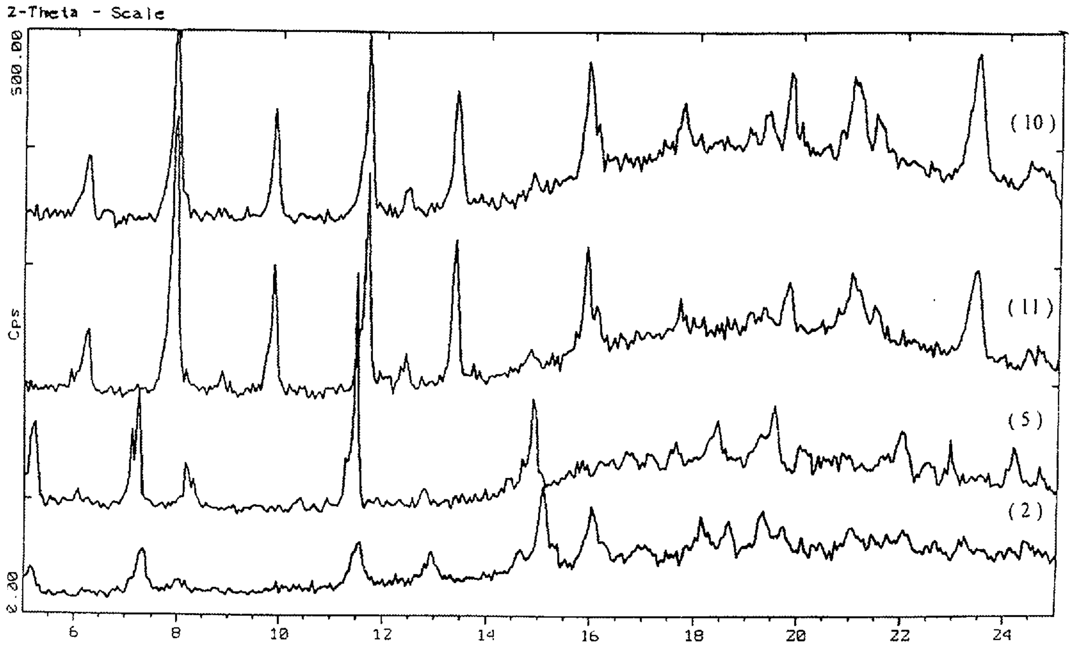

Figure 1. Diffraction patterns for products obtained during daunorubicin hydrochloride purification procedures by use of following solvents: chloroform (2), 1,1,2,2–tetrachloroethan (5), ethyl chloride (11), *trans*–1,2–dichloroethylene (10).

selected samples of daunorubicin hydrochloride are shown in Figure 1 and in Table 3.

Moreover, on the basis of relative intensities of diffraction signals, it was possible to estimate the regularity of the crystal lattice, *e.g.* samples crystallized from ethyl chloride or *trans* 1,2–dichlorethylene showed most intense diffractions, in contrast to the samples crystallized from 1,1,2,2–tetrachlorethylene or chloroform.

In comparison with the known procedures this method of purification has considerable advantages such as:

– higher yield (in the range of 82.3–86.2%),

– one step of purification procedure,

– lower content (≤0.1%) of solvents in final product, whereas according to the known methods this content, for example, in the case of using of chloroform, is in the range of 10.4–14.0%,

– shorter time of crystallization – no more than about 20 hours,

– smaller volume of solvents with respect to 1 g of crude daunorubicin hydrochloride (for example, according to patent application (5) it amounts 110 ml, whereas according to our method it amounts maximum 60 ml),

– shorter time of drying of the final product at temperature ≤ 35°C,

– shorter time of a whole process of purification, as a result of shorter time of crystallization and drying,

– possibility of pure product isolation from fermentation broth, after their initial purification.

This method is also more economical due to minor volume of solvents used, shorter time of crystallization as well as shorter time of drying in lower temperature. Moreover, daunorubicin hydrochloride obtained according to this method has excellent physico-chemical properties, including stability on storage and solubility in water and – what is very important – does not contain solvent molecules included in crystal lattice.

Acknowledgement

Authors would like to thank M.Sc. Bożena Tejchman from the Institute Biotechnology and Antibiotics for carrying out the analysis.

REFERENCES

1. Oszczapowicz I., Wąsowska M.: Farm. Pol. 55 (2), 80 (1999).
2. Patent USA No. 3875010.
3. Patent USA No. 3989598.
4. European Patent Application No. 306541 A.
5. International Patent Application No. 99/29708–A1.
6. European Pharmacopoeia, p. 706 (1997).
7. Angiuli R., Foresti E., Riva di Sanseverino L., Isaacs N., Kennard O., Morthrerwell W., Wampler D., New Biol. 234, 78 (1971).

Received: 28.11.2000