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Determination of cyclophosphamide and its metabolites in human plasma by high-performance liquid chromatography–mass spectrometry

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Abstract

A sensitive HPLC–MS method was developed for the simultaneous determination of cyclophosphamide and its metabolites 4-hydroxycyclophosphamide (aldocyclophosphamide), 4-ketocyclophosphamide, caboxyphosphamide and 3-dechloroethylifosfamide in human plasma. 4-Hydroxycyclophosphamide was converted with methylhydroxylamine to the stable methyloxime form. We used a solid-phase extraction with C_{18} cartridges followed by HPLC–MS with the single mass spectrometer SSQ 7000 of Finnigan. The limits of detection were 15 ng/ml for cyclophosphamide, 3-dechloroethylifosfamide and ketocyclophosphamide in each case and 30 ng/ml for carboxyphosphamide and 4-hydroxycyclophosphamide, respectively. First results of pharmacokinetics are shown.

Keywords: Cyclophosphamide

1. Introduction

Cyclophosphamide is an anticancer and immunosuppressive drug. Oxidation by hepatic microsomal mixed-function oxidase enzymes produces 4-hydroxycyclophosphamide, which exists in equilibrium with aldophosphamide. Aldophosphamide decomposes to phosphoramide mustard and acrolein. 4-hydroxycyclophosphamide is metabolized by a aldehyde oxidase to 4-ketocyclophosphamide and aldophosphamide is converted to carboxyphosphamide. Another way of detoxification of cyclophosphamide is the reaction involving conversion to 3-dechloroethylifosfamide [1].

Cyclophosphamide and its metabolites were determined by different methods [2–6]. 4-hydroxycyclophosphamide is very unstable and therefore derivatives are often formed. Many substances for the derivatization are used, for example *p*-nitrophenylhydrazine, semicarbazide, sodium cyanate and *o*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine [7–11]. An indirect fluorometric method measuring acrolein, a decomposition product of 4-hydroxycyclophosphamide/aldophosphamide, has been used for the quantitation of 4-hydroxycyclophosphamide/aldophosphamide [12]. This method showed high sensitivity with a detection limit of 5 ng/ml. For cyclophosphamide a very sensitive GC–MS method was described with a detection limit of 1 ng/ml [1,13]. This method also describes the determination

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of different metabolites of cyclophosphamide apart from 4-hydroxycyclophosphamide. The off-line combination of high pressure liquid chromatography and field desorption mass spectrometry has been used for the simultaneous determination of ketocyclophosphamide and carboxyphosphamide [14]. However, a sensitive method for the simultaneous measurement of cyclophosphamide and its metabolites 4-hydroxycyclophosphamide (aldophosphamide), carboxyphosphamide, ketocyclophosphamide and 3-dechloroethylifosfamide has not been described in the literature up to now. According to the ^{31}NMR method we derivatized 4-hydroxycyclophosphamide (aldophosphamide) with methylhydroxylamine to form the stable methyloxime of aldophosphamide [15]. Furthermore we could show that the other metabolites of cyclophosphamide and cyclophosphamide itself did not react with methylhydroxylamine.

2. Experimental

2.1. Chemicals

Ifosfamide, cyclophosphamide, 3-dechloroethylifosfamide, carboxyphosphamide, ketocyclophosphamide and 4-peroxycyclophosphamide were gifts from ASTA MEDICA AWD (Frankfurt am Main, Germany). Acetic acid, methanol (HPLC grade), ammonium acetate (A.C.S.) and acetonitrile (HPLC grade) were obtained from Baker. The substance methylhydroxylamine–hydrochloride was from Sigma.

2.2. Calibration and controls

2.2.1. Preparation of calibration samples and controls

Cyclophosphamide and its metabolites were separately weighed and dissolved in ammonium acetate solution. A stock solution of all substances was prepared from separate solutions. This was necessary because the concentrations of all the substances are very different. All solutions were cooled at 4°C. The calibration samples were produced with blank plasma and different amounts of the stock solution. In addition three controls were produced which were similar to the calibration samples. The substances were again weighed and a new stock solution was made. All aliquot samples were stored at −70°C.

2.2.2. Stock solution of internal standard

A solution was made from ammonium acetate and 1 mg ifosfamide in 1 ml. This solution was diluted with the same ammonium acetate solution, so that we kept an end concentration of 20 μg/ml. The ammonium acetate solution was 0.5 *N*.

2.2.3. Stock solution of derivatization

o-Methylhydroxylamine-hydrochloride was weighed and dissolved in doubly distilled water, to maintain an end concentration of 15 mg/ml. The solution was then neutralized with sodium hydroxide. This solution was stored at −24°C until derivatization.

2.3. Preparation of samples

Fifty microlitres of stock solution of methylhydroxylamine and 50 μl of stock solution of internal standard (1 μg ifosfamide) was added to 450 μl plasma. The derivatization of aldophosphamide with methylhydroxylamine was realized by heating to 50°C for 5 min. Three hundred and fifty mirolitres of 1.65%-ic acetic acid were added to the cooled samples. The whole sample was used for solid extraction with Li Chrolut^{R} solid-phase extraction cartridges RP18 from Merck. The cartridges were pretreated three times with 1 ml acetonitrile and 1 ml 0.5%-ic acetic acid. After the plasma was transferred to the cartridges these were washed twice with 0.5%-ic acetic acid. Then the cartridges were eluted with 3×1 ml acetonitrile. After evaporation with nitrogen the residues were stored at −70°C until determination.

2.4. Chromatography

The following devices were used: membrane degasser (TSP), pump consta Metric 4100 MS, autosampler Spectra system AS 3000 (both TSP) and a single mass spectrometer SSQ 7000 of Finnigan. A Ultrasep ES 100 Pharm RP18 (5 μm, 125×2 mm) column was used. The mobile phase consisted of 30% methanol and 70% 20 m*M* ammonium acetate

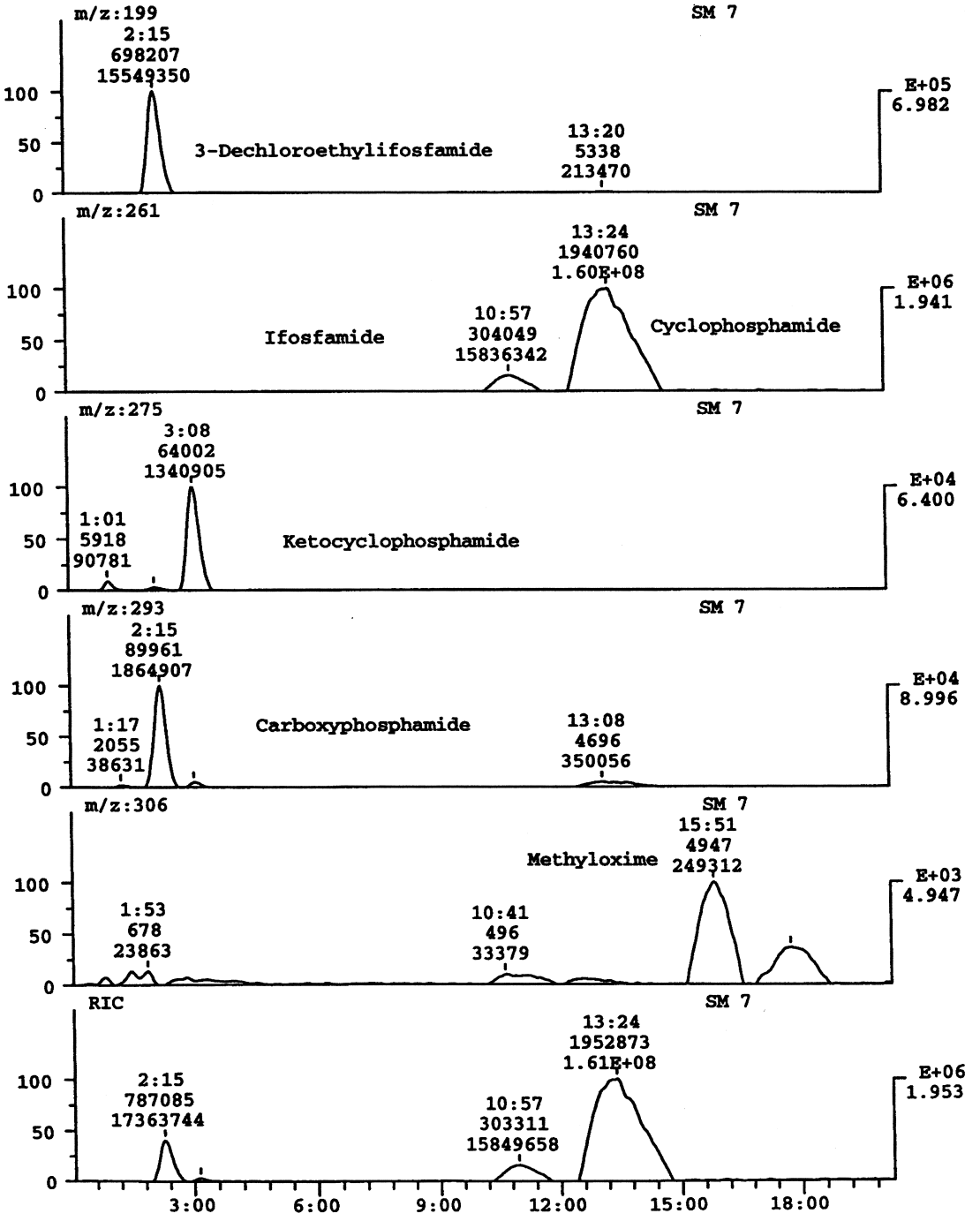

Fig. 1. Chromatogram of spiked plasma with cyclophospamide and its metabolites.

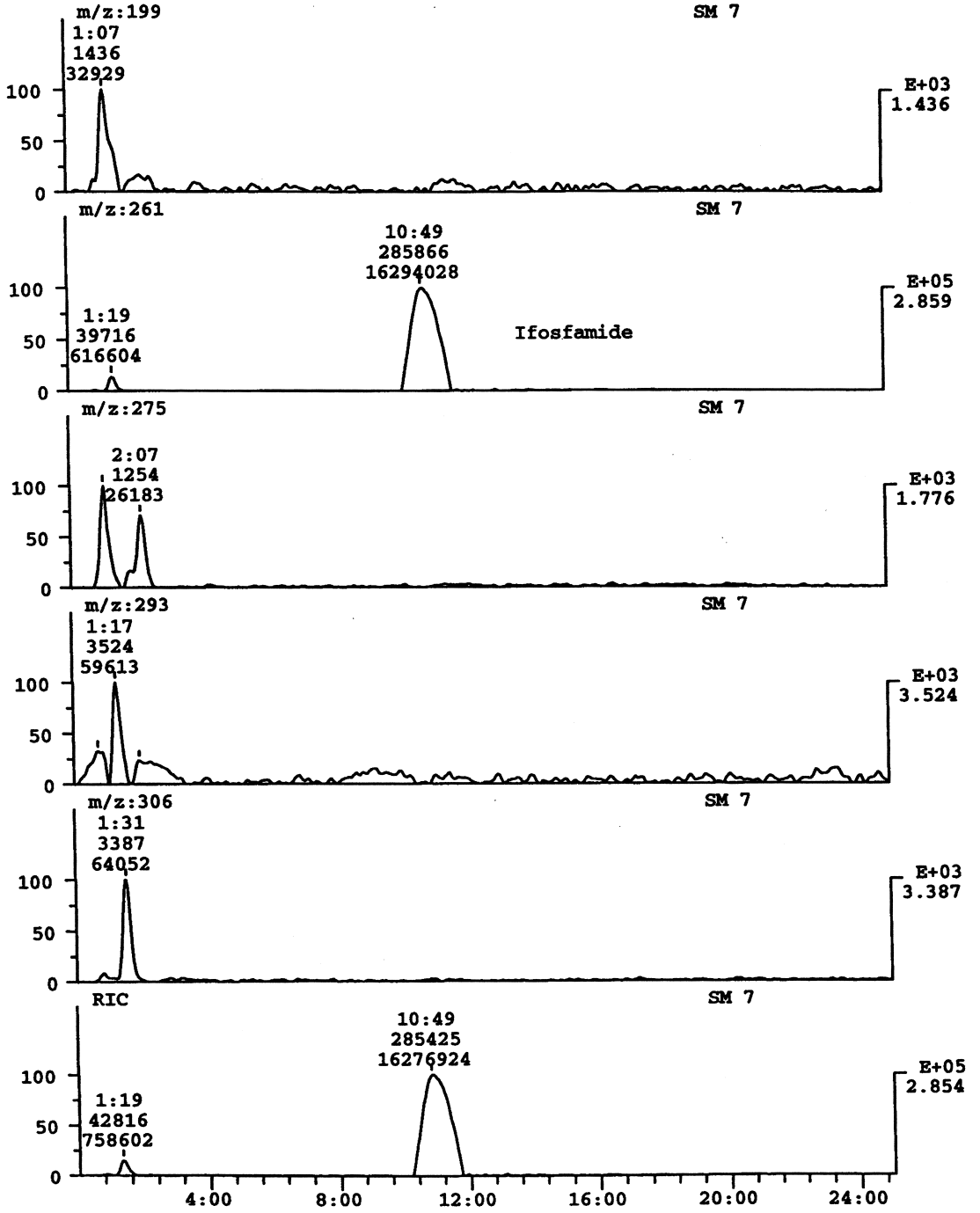

Fig. 2. Chromatogram of blank plasma with ifosfamide.

with 1‰ acetic acid. The flow-rate was 0.3 ml/min and the injection volume was 20 μl. The following parameters of mass spectrometry were used: 60 p.s.i. sheath gas (nitrogen), 15 p.s.i. auxillary gas, 10 V CID, 4.5 kV voltage of needle and 220°C temperature of capillary. Following mass tracks by selected ion mode (SIM) and positive mode were used: 199, 261, 275, 293 and 306 by 0.6 units and 2 s. The residues were diluted in 100 μl 30% methanol–70% 20 m*M* ammonium acetate (pH 7.4). Due to the instability of carboxyphosphamide the samples were measured within 2 h.

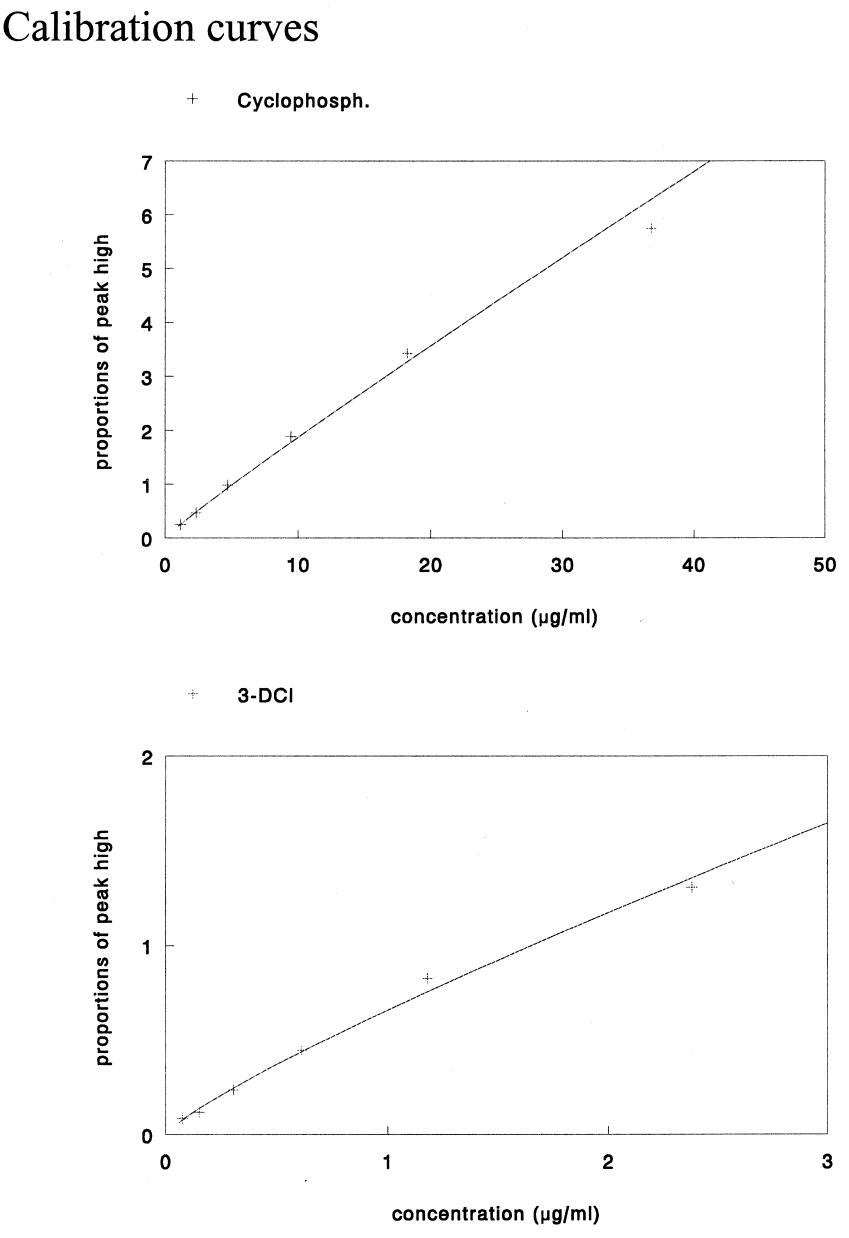

Fig. 3. Calibration curves of cyclophosphamide and 3-DCl.

Table 1
Intra assay and recovery rate[a]

	3-Dechloroethyl-ifosfamide	Carboxyphos-phamide	Ketocyclophos-phamide	Cyclo-phosphamide	4-Hydroxycyclo-phosphamide
A v (%)	2.8	3.9	3.4	8.7	6.2
A recovery rate	72.1	70.0	83.4	84.6	
A conc. μg/ml	2.032	3.599	2.343	27.584	0.6891
B v (%)	4.6	7.4	5.1	7.8	7.7
B recovery rate	76.3	60.8	88.0	84.7	
B conc. μg/ml	0.692	1.226	0.798	9.400	0.2348
C v (%)	11.4	14.5	10.9	10.6	
C recovery rate	77.1	70.0	88.4	66.3	
C conc. μg/ml	0.138	0.245	0.160	1.880	

[a] $n=10$; v Relative deviation (%) (within day); recovery rate in %.

3. Results and discussion

Fig. 1 shows a chromatogram of a spiked sample. The substances were determined as molecule ion (MH^+) in the following order: 3-dechloroethylifosfamide (199), carboxyphosphamide (293), ketocyclophosphamide (275), ifosfamide (261), cyclophosphamide (261) and 4-hydroxycyclophosphamide (aldophosphamide) as methyloxime (306). In Fig. 2 a chromatogram of a blank plasma with the internal standard ifosfamide is presented. No interferences were observed by single mass tracks. 3-Dechloroethylifosfamide has a similar retention time to carboxyphosphamide, but this substance shows no interferences to mass tracks 293, therefore the separation is enough. The separation between ifosfamide and cyclophosphamide by the mass track 261 is also sufficient. The limits of quantitation and of detection based on ten times noise and three times noise, respectively and are: 3-dechloroethylifosfamide, 50 ng/ml and 15 ng/ml; carboxyphosphamide, 100 ng/ml and 30 ng/ml; ketocyclophosphamide, 50 ng/ml and 15 ng/ml; cyclophosphamide, 50 ng/ml and 15 ng/ml; 4-hydroxycyclophosphamide 100 ng/ml and 30 ng/ml.

Fig. 3 shows the calibration curves. 3-Dechloroethylifosfamide and cyclophosphamide show a nonlinear behaviour in the used concentration ranges. For ketocyclophosphamide, carboxyphosphamide and 4-hydroxycyclophosphamide the concentration (c) and the measured intensity is a linear relationship. Typical calibration curves were: ketocyclophosphamide $\mathcal{V}=0.000137+0.0735\times c$ (μg/ml); $r=0.998$; carboxyphosphamide $\mathcal{V}=0.001683+0.078\times c$ (μg/ml); $r=0.998$ and 4-hydroxycyclophosphamide $\mathcal{V}=0.000052+0.0596\times c$ (μg/ml); $r=0.99993$. $\mathcal{V}$ is the peak high ratio.

The intra-assay and the recovery rate were determined using the controls A–C. Table 1 shows these values for cyclophosphamide and all its metabolites. The recovery rate of 4-hydroxycyclophosphamide cannot be determined because this compound is very unstable. This substance was determined as methyloxime of aldophosphamide. The deviation between days is greater than within day, because the sensitiveness of mass spectrometer

Table 2
Stability at room temperature (autosampler)

Time (h)	3-DCl (%)	Carboxyphos-phamide (%)	Ketocyclo-phosphamide (%)	Cyclo-phosphamide (%)	4-Hydroxy-cyclophosphamide (%)	Ifosfamide (%)
2	105.7	93.9	105.9	109.1	116.7	103.0
3	102.0	83.6	99.1	93.4	107.6	98.3
5	102.2	78.7	101.7	103.3	101.1	109.0
7	103.4	70.7	102.0	101.0	102.9	105.5
24	100.3	31.7	102.3	97.2	102.7	96.0

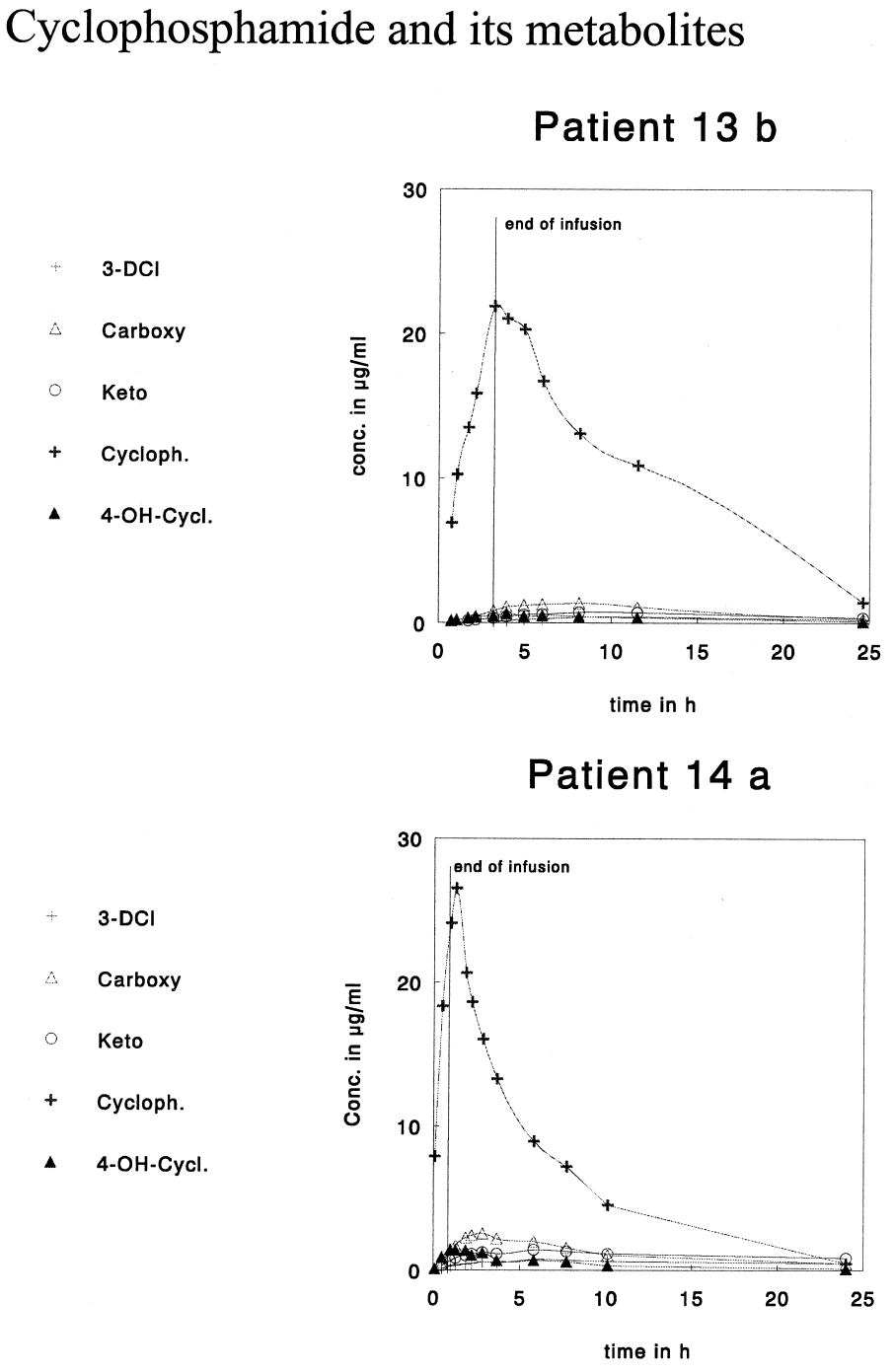

Fig. 4. Pharmacokinetics of cyclophosphamide and its metabolites of two patients after i.v. administration of 1100 mg cyclophosphamide.

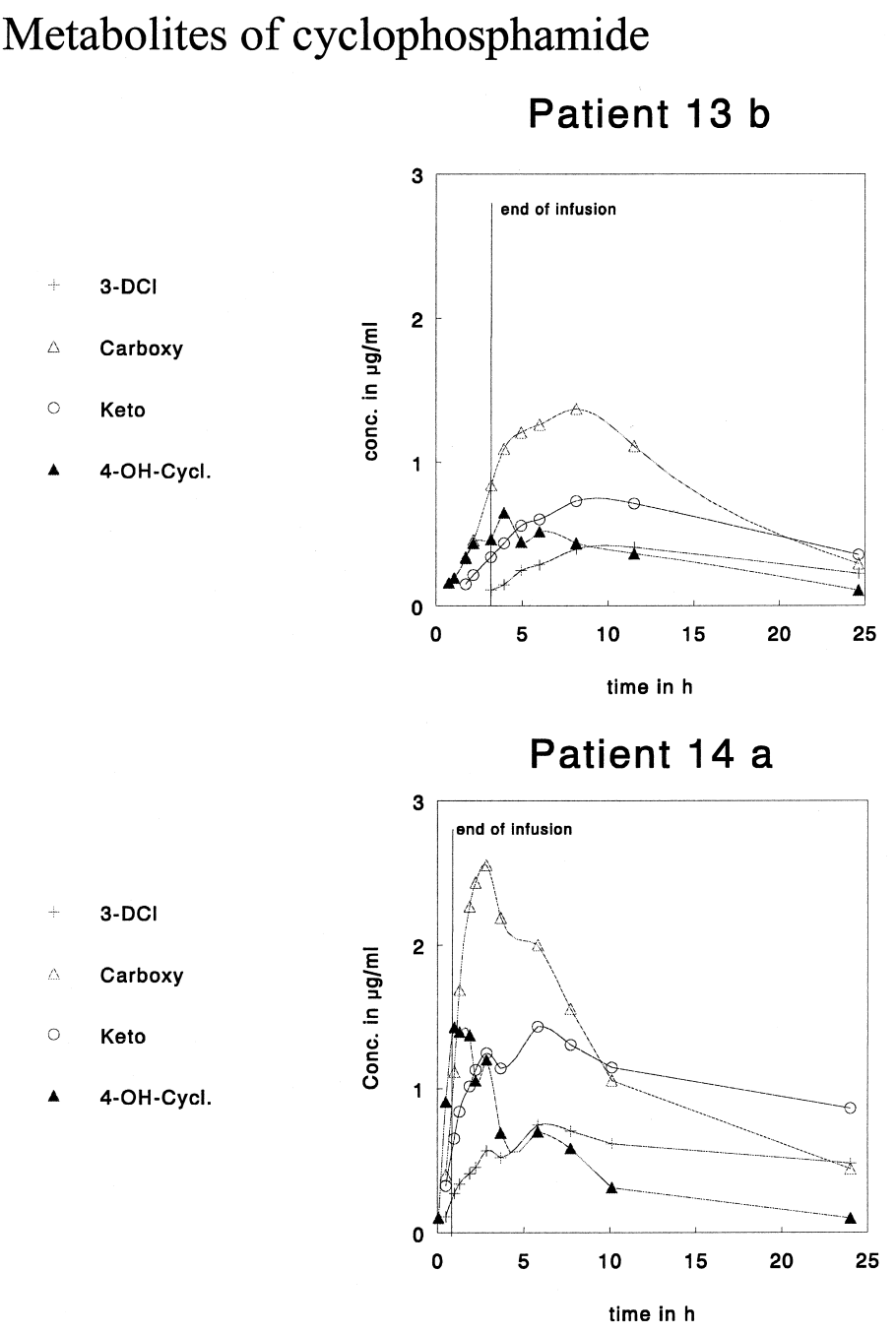

Fig. 5. Separate presentation of the pharmacokinetics of metabolites of two patients after i.v. administration of 1100 mg cyclophosphamide.

was relative strong changed. Therefore daily calibration curves were used for all substances with six measuring points. The coefficients of correlation r should be greater than 0.99. Three controls were used for the check of daily calibration curves by the measuring of patient samples.We used the confidence

intervals of Intra-assay as exclusion criterion. When the concentration of cyclophosphamide is higher than 40 μg/ml the patient samples and the samples of calibration should be diluted with 200 μl instead of 100 μl, because the intensities are in range of saturation.

Table 2 shows the stability of substances at room temperature. The standards (100%) were defrosted before the measurement. The substances were diluted in 30% methanol–70% 20 m*M* ammonium acetate (pH 7.4). Apart from carboxyphosphamide all other substances were stable. Carboxyphosphamide can be determined up to 2 h by storage in an autosampler. When stored longer than 2 h carboxyphosphamide decomposed more than 10 percent of the starting concentration.

To investigate the stability of 4-hydroxycyclophosphamide blood was taken from patients and then centrifuged at 4°C. This plasma was stored at 4°C until derivatization. After 30 min, 60 min, 90 min and 120 min the plasma samples were derivatized. The measured values of methyloximes were similar over this period. 4-Hydroxycyclophosphamide is stable under these conditions.

Fig. 4 shows the kinetics of cyclophosphamide and its metabolites from two patients. In Fig. 5 only the metabolites of these patients are presented. The decrease in concentration of cyclophosphamide and 4-hydroxycyclophosphamide is similar. Only the absolute values distinguish strong from each other.

The purpose of our work was the development of a sensitive method for the simultaneous determination of cyclophosphamide and all its metabolites in human plasma.

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