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DETERMINATION OF HYDROXYPROPYL-β-CYCLODEXTRIN IN PLASMA AND URINE BY SIZE-EXCLUSION CHROMATOGRAPHY WITH POST-COLUMN COMPLEXATION

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SUMMARY

The analytical method described here provides the appropriate sensitivity and selectivity for the determination of unlabelled hydroxypropyl-β-cyclodextrin as a parenteral carrier in pharmaceutical formulations. The method may also be used in clinical trials evaluating the fate and pharmacokinetic profile of this compound, which was isolated from the biological matrix by solid-phase extraction with Bond Elut C_{18} cartridges. The lack of uniformity of the product was circumvented by the use of a size-exclusion chromatographic column. An indirect colorimetric complexation method was used for detection. The detection limit was 0.1 μg per 2 ml of biological fluid and the extraction recovery was sufficient (78%).

INTRODUCTION

Cyclodextrins are able to form inclusion complexes by taking up various lipophilic guest molecules. The cavity of β-cyclodextrin (β-CD) seems to be most favourable for many pharmaceuticals but its poor solubility in water [1.8% (w/v) at 25°C] is a serious drawback for solubilization. As this relatively low water solubility is due to the formation of rather stable intramolecular hydrogen bonds, partial alkylation or acylation of the primary hydroxyl functions highly enhances the solubility without a loss of inclusion capacity [1,2]. The solubility of 2-hydroxypropyl-β-CD (HP-β-CD) is more than 30% (w/v) at 25°C, but the product is inhomogeneous because of the large number of hydroxyl groups (21 for β-CD), which makes isomerism possible. The molar substitution (MS) value indicates the degree of alkylation. An MS value of 0.4 was found to be optimal for the inclusion complex to be used as a parenteral formulation, because an alkylated β-CD of higher value might be precipitated by heat sterilization [1] and, owing to the marked surface activity, the haemolytical effect can be enhanced [3].

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The high-performance liquid chromatographic (HPLC) columns for the separation of homogeneous CDs could not be applied in this case because HP-β-CD showed incomplete resolution in the form of broad peaks. Thus size-exclusion chromatography was used on a column of relatively low-molecular-mass resolution, which obviated the lack of uniformity.

Despite the increasing importance of the application of various CDs in pharmacological use [4], only a few publications deal with the detection of CDs in biological matrices. The absence of chromophoric and electroactive groups prevents direct spectrophotometric or amperometric detection. From a pharmacokinetic point of view, the refractive index detector [5] is not sensitive, and the detection of ^{14}C-labelled CDs is too complicated for routine usage [6]. An excellent idea for the determination of β-CD was published by Vikmon [7], which is based on the high stability of the inclusion complex with alkaline phenolphthalein. The colour intensity was proportionally decreased as the concentration of CDs increased. The method was applied for quality control of different batches in CD production. Frijlink et al. [8] showed that post-column complexation is a sensitive detection method in HPLC for pharmacokinetic studies. This indirect colorimetric complexation method was also found to be applicable for detection of HP-β-CD.

The isolation of HP-β-CD from the biological matrix was carried out by solid-phase extraction with Bond Elut C_{18} cartridges.

Among the CDs there are a number of differently alkylated analogues of β-CD, but the HP-β-CD derivative appears to be the most promising candidate for a parenteral carrier because of its complexation power, solubility and non-toxic character [2,9].

EXPERIMENTAL

Chemicals

HP-β-CD was purchased from Chinoin (Budapest, Hungary) with an MS value of 0.38 and an average molecular mass of 1229. The product was free from non-alkylated β-CD, as checked by HPLC. Standard solutions of 0.25 μg per 100 μl up to 500 μg per 100 μl were prepared with water, obtained from a Milli-Q reagent water purifying system (Millipore, Molsheim, France). Other chemicals were of the best grade commercially available. The complexation reagent was made by adding 10 ml of 0.006 *M* methanolic phenolphthalein to 990 ml of 0.008 *M* aqueous sodium carbonate, and adjusted to pH 10.5 by 1 *M* sodium hydroxide. Bond Elut C_{18} cartridges (solvent mass/column volume = 500 mg per 2.8 ml) were purchased from Analytichem (Harbor City, CA, U.S.A.).

Chromatographic conditions

The HPLC equipment used consisted of two LC-6A pumps [Shimadzu (Europa), Duisburg, F.R.G.] One pump (for delivering alkaline phenophthalein) was coupled with a pulse dampener (Type PD-1; Gynkotek, Munich, F.R.G.) and a pre-column (packed with 5-μm chemically bonded ODS). The flow-rate of 0.8 ml/min resulted in a back-pressure of 2.2 Mpa (300 p.s.i.). The second pump was

used for the analytical column .The spectrophotometric detector (SPD-6AV, Shimadzu) operated at 553 nm. Injections were made by an autosampler (SIL-6A, Shimadzu) with a controller. A Spectra-Physics Model 4290 integrator linked to a LABNET data system (Spectra-Physics, Darmstadt, F.R.G.) was used for quantitative determinations. Separations were carried out on a Shim-Pack Diol-150 (25 cm×7.9 mm I.D.) column packed with fully porous, spherical silica particles (5 μm) to which diol radicals are chemically bonded. The mobile phase was water with a flow-rate of 1.2 ml/min, resulting in a back-pressure of 7 Mpa (1000 p.s.i.). The retention time for HP-β-CD was 7.6 min. Complete post-column mixing was achieved by stainless-steel capillary tubing (1.5 m×0.5 mm I.D.; 1.59 mm O.D.) between the Tee connector and the detector, as suggested by Frijlink et al. [8].

Extraction method

Bond Elut columns, attached to a Vac Elut processing station (Analytichem) were conditioned by passing 2.5 ml of water, 1.25 ml of methanol and finally 2.5 ml of water. Samples of 1 ml (or more) of plasma or urine were then quantitatively applied. After the C_{18} cartridges had been washed with 2.5 ml of water they were dried by passing air through them. Retained CD was collected by 1.25 ml of 33% (v/v) methanolic water. Solutions were evaporated under nitrogen at 60°C. Dried extracts were dissolved in 150 μl of water, and 100 μl were injected into the HPLC column.

Calibration and calculation

Using HP-β-CD standard solutions, separate series of blank plasma and urine samples (1 ml) were spiked at concentrations ranging from 0.5 to 200 μg/ml. These calibration samples were extracted as described above.

Unknown sample concentrations were calculated by determining the peak areas of HP-β-CD and comparing these areas with the standard curve obtained from analysis of the calibration samples.

Pharmacokinetic experiment

Three male Wistar rats (mean weight 340 g) were anaesthetized with pentobarbital, then injected intravenously (caudal vein) with an 80 mg/ml solution of HP-β-CD in physiological sodium chloride, at a dose of 80 mg/kg. Blood samples of ca 0.25 ml were taken from the cannulated femoral artery at appropriate time intervals. Pooled samples were centrifuged and the plasma was analysed as described above.

RESULTS AND DISCUSSION

The overall recovery of the extraction procedure for HP-β-CD from 1 ml of control plasma was 78% (see Table I). Representative chromatograms (Fig. 1) of standard, extracts from blank plasma and blank plasma spiked with 5 μg/ml HP-β-CD and plasma of treated rats show that the criteria of sensitivity and purity were met. The method was validated by using spiked plasma samples. Rea-

TABLE I

RECOVERY STUDY OF HYDROXYPROPYL-β-CYCLODEXTRIN IN SPIKED PLASMA SAMPLES

Theoretical concentration (μg/ml)	Recovery (mean ± S.D.) (%)	n	Coefficient of variation (%)
0.5	86.7 ± 1.6	3	1.8
1.0	82.3 ± 1.3	3	1.5
2.5	78.0 ± 7.8	5	10.0
5.0	75.2 ± 3.6	5	4.8
10.0	72.3 ± 6.1	5	8.4
25.0	78.5 ± 3.0	5	3.8
50.0	71.2 ± 7.9	5	11.2
100.0	75.5 ± 2.3	5	3.0
200.0	82.7 ± 1.7	5	2.1
Mean	78.0		

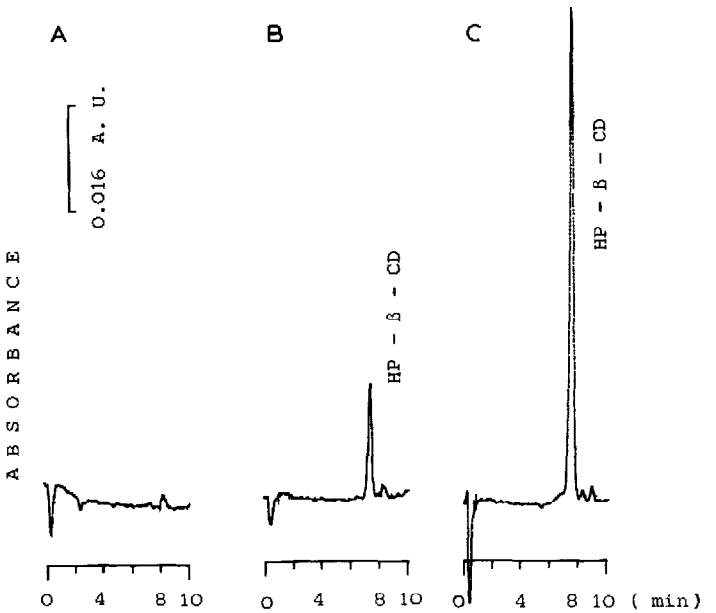

Fig. 1. Chromatograms of extracts from (A) blank control plasma, (B) control plasma spiked with 5 μg/ml HP-β-CD and (C) plasma from rats 30 min after an intravenous dose of 80 mg/kg (plasma level, 54 μg/ml).

sonable linearity and a negligible intercept were found routinely. Least-squares regression analysis yielded the regression equation $y=0.9182x+4.9168$ ($r^2=0.9948$). The accuracy and precision of the method are presented in Table II.

It is well known that CDs (α, β and γ) form stable inclusion complexes with phenolphthalein in aqueous solution at pH 10.5 [7]. Because the central cavities

TABLE II

ACCURACY AND PRECISION OF THE HPLC METHOD FOR THE DETERMINATION OF HYDROXYPROPYL-β-CYCLODEXTRIN IN PLASMA

Theoretical concentration (μg/ml)	Observed concentration (mean ± S.D.) (μg/ml)	n	Coefficient of variation (%)	Accuracy (%)
0.5	0.43 ± 0.04	5	8.0	86.0
1.0	0.98 ± 0.04	6	4.0	98.0
2.5	2.52 ± 0.22	6	8.8	108.0
5.0	4.77 ± 1.1	11	22.0	95.4
10.0	10.30 ± 2.2	11	22.0	103.0
25.0	23.83 ± 4.8	10	19.2	95.3
50.0	53.52 ± 8.2	10	16.4	107.0
100.0	96.24 ± 5.6	6	5.6	96.2
200.0	210.14 ± 12.6	5	6.3	105.1

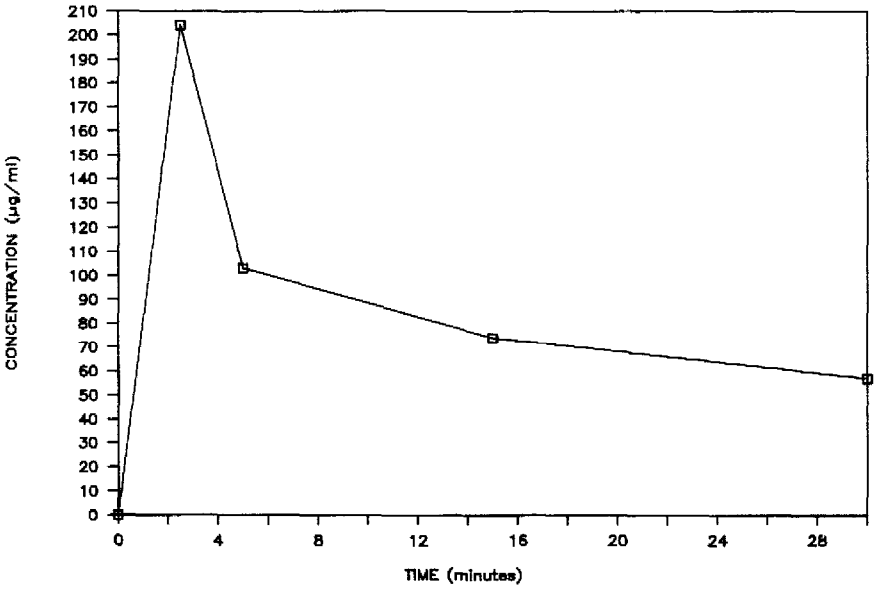

Fig. 2. Plasma levels in rats after intravenous administration of 80 mg/kg HP-β-CD.

of β-CD and HP-β-CD are equivalent, the inclusion characteristics of the two compounds with phenolphthalein are identical too. Because of the specificity of this detection a direct spectrophotometric determination of HP-β-CD in plasma was attempted after protein precipitation. Using different precipitation reagents (sulphosalicylic acid, perchloric acid and trichloroacetic acid), the solutions were neutralized and a complexing mixture was added. However, the flat slope of the small concentration steps plotted against absorbance showed rather low sensitivity that made direct assay for biological matrices problematic. It was obvious that the HPLC method for β-CD and γ-CD described by Frijlink et al. [8] for pharmacokinetic measurements is superior to direct spectrophotometry.

The specificity of the reaction was later proved by injecting non-complexing agents, such as polyethyleneglycol of different molecular mass (8000, 6000 and 3300), into the chromatographic system described above. The responses of these substances obtained by an injection of 1500 μg of each are similar to that of 2.5 μg (ratio 600:1) of HP-β-CD.

The HPLC separation of chemically uniform CDs, such as α-, β- and γ-CDs, heptakis-(2,6-di-O-methyl)-β-CD or branched CDs, has been widely discussed and described by Koizumi et al. [10] for reversed-phase, by Koizumi et al. [5] and Gerloczy et al. [11] for amino-bonded silica, by Zsadon et al. [12] for μBondapak–carbohydrate and Brauns [1] for aminopropyl-bonded silica, by Koizumi et al. [5] for vinyl alcohol polymer (Asaphiak GS-320) and by Frijlink et al. [8] for phenyl-modified silica columns.

Brauns [1] pointed out that HP-β-CD with a low MS value gave a broad and unresolved peak formation on aminopropyl-bonded silica owing to unresolved isomers. Our aim was to obtain these fractions as a single peak, making quantitative evaluation possible.

The Shim-Pack Diol 150 column is specially designed for high-performance aqueous size-exclusion chromatography with an average pore diameter of 150 Å. Taking the average molecular mass (1229) of HP-β-CD into account, the retention time (7.6 min) achieved with a flow-rate of 1.2 ml/min correlates fairly well with the extrapolated retention times of the calibration curves obtained with peptides or polyethyleneglycols. Although α-, β- and γ-CDs can also be detected, the separation was not satisfactory because of the relatively low differences between the molecular masses. However, this was not the purpose of the study.

It is an important aspect of the assay that water is used as a mobile phase with no change of pH. We found that an injection volume of as little as 100 μl, varying the pH in the mobile phase by two units in either direction, was enough to disturb the delicate balance between water (as mobile phase) and alkaline phenolphthalein reagent. It is preferable to apply the preconcentration suggested, using C_{18} cartridges, because the aqueous washout followed by dissolution in water ensures a neutral and clean extract. The sample preparation described avoids the disadvantages, such as dilution of matrix and neutralization, required by the acidic protein precipitation discussed by Frijlink et al. [8]. Moreover, it improves the detection limit and it offers an opportunity to use various amounts of biological matrices. The overall recovery (see Table I) of 78% is acceptable. Uekama and Irie [4] reported that some β-CDs (dimethyl-β-, trimethyl-β- and β-CD), owing to their inclusion properties, can interact with phospholipids, cholesterol and proteins. For this reason, we checked whether the recovery could be increased by protein precipitation followed by preconcentration. Using sulphosalicylic and trichloroacetic acids for this purpose we were unable to improve the recovery and purity significantly. An attempt was made to use C_{18} cartridges in an on-line arrangement, employing a column-switching module (Gynkotek Type SE-2). In this case, methanol–water has to be used as a mobile phase to remove retained CD from the surface of the C_{18} cartridges after aqueous washout. However, the

presence of ca. 10% (v/v) methanol (or other organic solvents) disturbed the complexation process [8], and above 30% there is no reaction at all.

In our method, the use of an internal standard was avoided because the requirements for its application were not fulfilled. The special detection, the adequate difference in molecular mass and the appropriate complexation properties made it difficult to find a similar chemical structure. Moreover, the internal standard technique does not necessarily improve the precision of an assay [13]. The autosampler used has a precision of injection better than 0.3%, which is a negligible contribution to the overall reproducibility. The coefficient of variation and the linearity over the concentration range 0.5–200 μg/ml show that the external standardization provides a reasonable quantification.

We did not observe the appearance of gas bubbles in the chromatographic set-up. Furthermore, ambient temperature was used, because the elevated temperature suggested by Frijlink et al. [8] has no influence on the sensitivity of complexation. The noisy baseline [8] could be effectively improved by inserting a pulse dampener and a small precolumn into the phenolphthalein line; this resulted in only a minimal drop of the back-pressure, as mentioned above.

Our preliminary pharmacokinetic experiment in rats was merely intended to demonstrate the applicability of the analytical assay (Fig. 2). Derivation of the final pharmacokinetic parameters would require a large number of animals and sampling points.

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