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Determination of the anticancer drug prospidin in human tissue by high-performance capillary electrophoresis using derivatization

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Abstract

A high-performance capillary electrophoresis (HPCE) method is described for the quantitative determination of the anticancer drug prospidin in human tissue after its derivatization with diethyldithiocarbamic acid sodium salt (DDTC). It was found that absorption of prospidin and its derivatives on the capillary wall due to the strong positive charge in the drug molecules could be eliminated by increasing the methanol content in the run buffer up to 50% and increasing the pH value up to 11.2. While studying the conditions of the interaction between prospidin and DDTC, a molar excess of the latter of 1:9 and 1.5 h of reaction time were found to be enough for complete derivatization. Sample preparation included homogenization of freshly cut papilloma species and deproteinization by methanol addition. Detection was by ultraviolet (UV) absorption at 254 nm. Due to its speed and high performance in separation, HPCE was found to be well suited for the fast checking of drug therapy in clinical practice.

Keywords: Prospidin

1. Introduction

The anticancer drug prospidin or N,N‴-bis-(γ-Cl-β-oxypropyl)–N,N″-dispyrotripiperazinium dichloride, is widely used in the chemotherapy of cancer diseases in clinical practice [1,2]. Like some of its analogues, which were synthesized earlier (e.g. melphalan), it is a derivative of nitrogen mustard. However, its general anticancer activity is significantly less toxic for human organisms. This is due mainly to the features of its chemical structure: the presence of hydroxy groups and longer aliphatic chains between Cl and N atoms leads to a reduction of its reactivity.

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Petersburg Institute of Pediatrics, prospidin was introduced in cases of infant papillomatosis, and therefore it became necessary to develop a method for the fast determination of prospidin in human tissue. Several earlier papers [3,4] had related the detection of some analogues of prospidin colorimetrically by nitrobenzylpyridine, but because of the laborious procedure involved and low efficiency of derivatization, it is not used in practice. Recently, Cummings et al. [5] described the determination of some reactive nitrogen mustard anticancer drugs in plasma by HPLC after its derivatization by the strongly nucleophilic reagent diethyldithiocarbamic acid (DDTC) to produce an adduct suitable for UV detection.

During the last few years high-performance capillary electrophoresis (HPCE) [6,7] has been applied to the analysis of many different substances in medicine [8,9], molecular biology [10], organic chemistry [11], forensic science [12] and ecology [13]. The development of HPCE separation procedure for the analysis of prospidin-like compounds (which have a strong positive charge at the quaternary nitrogen atom) is interesting. The presence of the charge causes electrostatic absorption of the compound on the negatively charged capillary wall, thus resulting in poor separation reproducibility. The use of specially coated silica capillaries can eliminate absorption [14,15]. Another possibility is to use uncoated capillaries but running a buffer with a very low pH value [16]. In that study absorption was eliminated, but the analysis time increased significantly (up to 40 min). Previously, we developed a general approach to the analysis of such compounds by HPCE using standard, uncoated, silica capillaries [17].

Based on this approach a quantitative determination of the anticancer drug prospidin in human tissue by HPCE has been developed. In the present paper we describe this method for the first time, including also drug derivatization by the UV-absorbing reagent, DDTC. Optimal conditions of sample preparation and analysis are discussed.

2. Experimental

2.1. Materials

Prospidin dihydrochloride as a lyophilized powder was obtained from Moschimpharmpreparat. DDTC (sodium salt) was from Reachim, Russia. Human papilloma species were donated by the Chair of Otolaryngology of the St. Petersburg Institute of Pediatrics. Methanol was HPLC-reagent grade. All other chemicals were of the highest grade available commercially.

2.2. Instrumentation

The capillary electrophoresis system consists of the following units: a high-voltage unit with constant voltage regulation (voltage range 2–20 kV) and UV detector (fixed wavelength 254 nm) which were from the Scientific–Industrial Ecological Centre Insovt. The separation and sample introduction were carried out in the positive polarity mode, i.e. the anodic reservoir served as a high-voltage output (injection end). Quartz capillary of the following size was used: 50 cm × 70 μm I.D. The length to the detector was 42 cm. The polyimide coating at the detector window was removed by flaming followed by a methanol wash. The capillary was conditioned by aspirating with 0.5 *M* sodium hydroxide–methanol (1:1) for 10 min, water for 10 min and the running buffer for 10 min. The running buffer was prepared by combining 50 parts of 20 m*M* borate, pH 11.2, with 50 parts of methanol. Injections by electroosmotic flow of 15 s were used for all HPCE studies.

2.3. Methods

In all studies unless otherwise mentioned, the reaction of prospidin derivatization by DDTC was carried out under the following conditions: to the prospidin solution in water was added the necessary quantity of DDTC solution in 0.05 *M* sodium hydroxide. The mixture was then incubated for 90 min at 37°C. At the end of incubation a cloudy precipitate formed. Then one part

of the reaction mixture (vigorously stirred immediately before use) was combined with four parts of methanol. The final solution was homogeneous. Immediately before applying to HPCE, each sample was combined with an equal volume of methanol.

2.4. Sample preparation

Samples of papilloma were homogenized and mixed with an equal volume of deionized, twice-distilled water. Then 200 μl of homogenate was added to 600 μl of methanol. After incubation for 1 h at room temperature, the protein precipitate was separated by centrifugation. The 150 μl of deproteinized solution was mixed with 100 μl of DDTC solution in 0.05 *M* sodium hydroxide at a concentration of 2 mg/ml. The sample was incubated at 37°C for 90 min and combined with an equal volume of methanol. After that the sample was ready for analysis by HPCE.

3. Results and discussion

3.1. Conditions selection for HPCE analyses

Due to the negatively charged inner wall of the uncoated silica capillary, some difficulties may arise when analysing prospidin-like compounds, which have a strong positive charge. These difficulties are mainly associated with absorption of substances on the capillary wall. Absorption could be decreased by the addition of an appropriate detergent (e.g. sodium dodecyl sulphate, SDS) in the buffer [18], which leads to the formation of micelles. In this case it was not useful because prospidin became water-insoluble after ionic binding with SDS.

The first HPCE separations were done with a buffer with a pH value of 8.5 and 10% methanol, but strong absorption was observed under these conditions. At pH 9.5 the absorption did not change. Only at pH 11.2 did it diminish significantly, but the effect of peak tailing was still observed on the electropherograms. For the complete elimination of absorption, we had to increase the methanol content of the buffer to 50%. This can be explained particularly by the increased electroosmotic flow when using a buffer with a higher pH value. Besides, increasing the ratio of methanol–water up to 1:1 in buffer leads to an increase in the solubility of the analyte which, in turn, also eliminates absorption.

It was shown that increasing the methanol content in the sample solution over its concentration in buffer increases the sensitivity of detection (unpublished results). This can be explained by the so-called "concentrating" effect.

3.2. Studies on the conditions of prospidin derivatization

UV detection is the most commonly used method in HPCE models, but prospidin cannot absorb UV light. This leads to the necessity of its derivatization by an appropriate UV-absorbing reagent. Earlier, DDTC was found to be well suited for the reaction with reactive nitrogen mustard derivatives [5]. With some alteration the same procedure was used for prospidin derivatization (Fig. 1).

For a detailed elaboration of the derivatization procedure, the optimal molar ratio of reagents in the reaction mixture and required reaction time were determined. Results of HPCE separation of reaction mixtures with different molar ratios of prospidin and DDTC are shown in Fig. 2. When the molar ratio was 1:1, two peaks were observed which apparently could be identified as mono- (peak N1) and disubstituted (peak N2) derivatives of prospidin. When the molar ratio was raised to 1:6, the yield of disubstituted prospidin significantly increased, followed by a decrease and then disappearance of the monosubstituted derivative. Such an identification of peaks parallels the situation of poles in our HPCE model (negative pole was at the detector). For such an electrode position it is obvious that monosubstituted prospidin will reach the detector first.

The results of the studies on the required reaction time are shown in Fig. 3. Samples for HPCE were taken every 10 min. The appearance

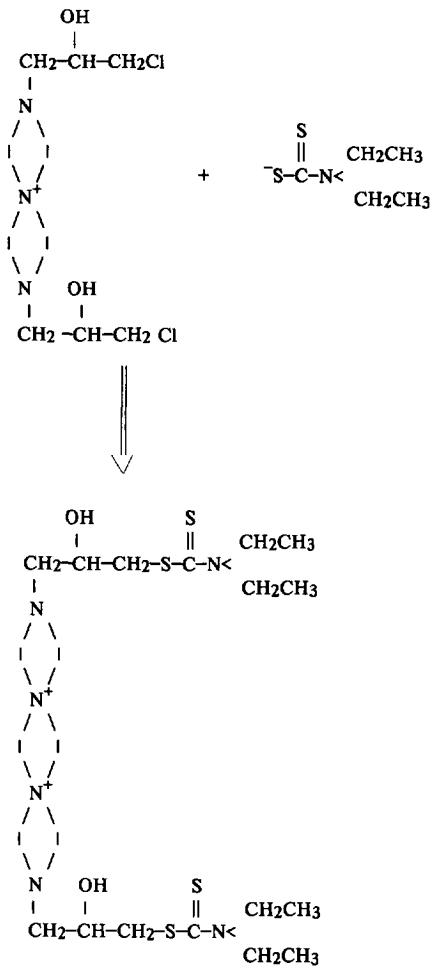

Fig. 1. Proposed reaction mechanism of prospidin with DDTC.

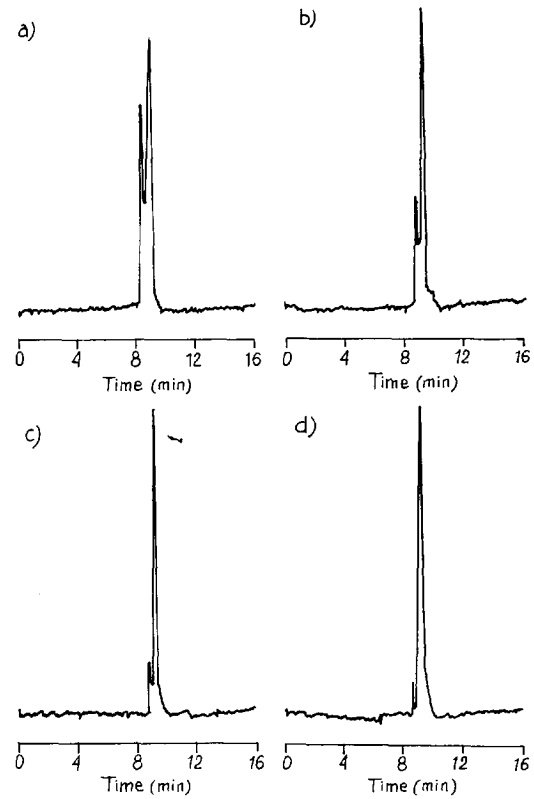

Fig. 2. HPCE separation of reaction products after derivatization with the following molar ratio of prospidin/DDTC: (a) 1:1, (b) 1:3, (c) 1:4, (d) 1:6. Conditions of separation: uncoated silica capillary 50 cm × 70 μm I.D.; voltage 14 kV; temperature 20°C; buffer 20 m*M* borate–50% methanol, pH 11.2; detector wavelength 254 nm.

of prospidin derivatives is observed only after 20 min of exposure. Despite the great excess of DDTC (the molar ratio is 1:9) at the beginning of the reaction, mainly the monosubstituted derivative is formed. After increasing the reaction time, disubstituted prospidin becomes the major component of the reaction mixture. After 1 h incubation monosubstituted prospidin disappears due to its complete conversion to the disubstituted form. The results obtained suggest that 1.5 h is enough to complete the derivatization of prospidin.

3.3. Determination of prospidin in papilloma homogenate

For the quantitative determination of prospidin in a homogenate of freshly cut papilloma, calibration curve data were obtained which reflected the dependence of the relative peak area (the peak area/retention time ratio) upon the drug concentration. Over the studied range of concentrations (1–50 μg/ml), the dependence was linear with a correlation coefficient of 0.997. The results obtained are shown in Table 1. Known amounts of prospidin were added to non-prospidin-treated papilloma homogenate. After

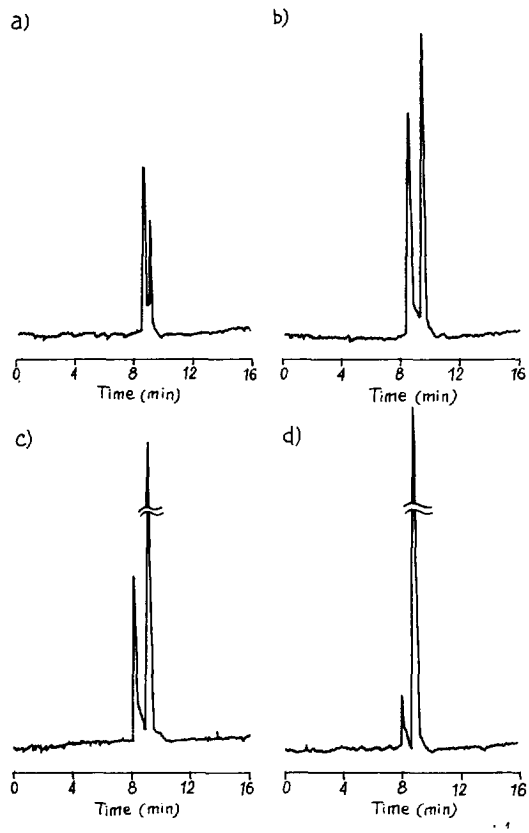

Fig. 3. HPCE separation of reaction products after derivatization for the following reaction times: (a) 20 min, (b) 30 min, (c) 40 min, (d) 60 min. Conditions: see Fig. 2.

Table 1
Standard calibration curve data for the disubstituted DDTC adducts of prospidin

Drug concentration (μg/ml)	Relative peak area
50	9.918
30	5.772
20	3.914
10	1.958
5	0.982

Approximation line, $y = -0.403 + 1.821x$; disp. after approx., 0.589. y = integrated peak area, x = drug concentration (μg/ml).

deproteinization equal amounts of DDTC were added to the solutions. Following the usual sample preparation, all five solutions were analyzed by HPCE.

On the electropherograms of all these samples (Fig. 4), the main peak of disubstituted prospidin (peak N1, retention time $t = 9.2$ min), a peak of unknown compound (peak N2, $t = 11.4$ min) and one which was previously identified as DDTC (peak N3, $t = 16.5$ min) were observed. Peak N2 was not observed on the electropherograms of prospidin–DDTC reaction mixtures studied for their interaction in buffer solutions as well as on the electropherograms of the mixture of non-prospidin-treated homogenate and DDTC. HPCE separation of prospidin-treated homogenate without DDTC derivatization also shows no peak N2. The ratio of relative peak areas N2/N1 in all samples was approximately equal (0.15–0.20), which suggests that in every case an equal part of the whole amount of the drug reacts with the homogenate components (apparently with thiols) at only one of the two Cl atoms. After DDTC treatment monosubstituted prospidin reacts with it at the second Cl atom, and thus peak N2 appears.

In this study we did not have to determine the

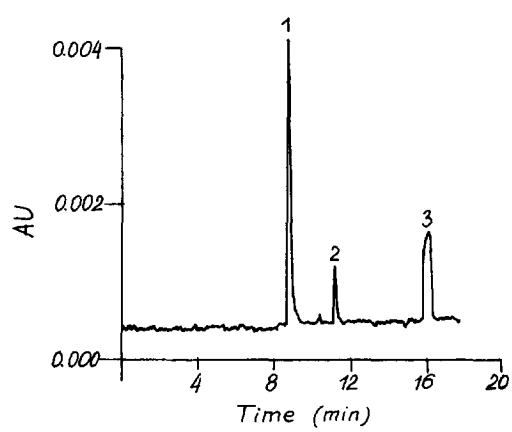

Fig. 4. HPCE separation of papilloma homogenate after prospidin addition and derivatization procedure for calibration of analysis. Conditions: see Fig. 2. Concentration of prospidin: 50 μg/ml. For key, see text.

total amount of the drug together with its metabolites, which is necessary, e.g., in the case of doping control. Here it was sufficient to confirm the presence of prospidin directly in papilloma and to evaluate the amount of its native form, which had not bound with the components of homogenate. Thus, we could disregard the presence of drug derivative, which gave peak N2.

The calibration curve data, shown in Table 1, do not consider this adduct and demonstrate only the dependence of relative peak area N1 upon the drug concentration.

3.4. Validation

Intra-day and inter-day precision of peak areas and retention times were tested over the same concentration range as for the calibration of analysis (5.0–50.0 μg/ml). The results obtained, which were expressed in terms of coefficient of variation (C.V.), were 1.8–2.8% for the intra-day assay and 2.0–3.1% for the inter-day assay (for the retention time). As shown in Table 2, the precision for the peak areas was 2.8–3.5% for the intra-day assay and 3.0–3.8% for the inter-day assay. The limit of detection was determined by finding the concentration of prospidin at which the peak area had a signal-to-noise (S/N) ratio of 3. It was found to be 1 μg/ml when analysed under the above-mentioned conditions.

3.5. Estimation of the stability of prospidin

To eliminate the potential underestimation of the drug due to its probable chemical destruction in tissue during sample preparation, the stability of prospidin was investigated. To this end, known amounts of drug were added to non-prospidin-treated papilloma homogenate, and the reaction mixture was incubated for 12 h at 37°C. Samples were taken 3 times, after 4, 8, and 12 h incubation. After the reaction with DDTC and usual sample preparation, the drug concentration was determined. It was found that under these conditions the loss of prospidin calculated from peak area N1 was 15% after 12 h incubation. This indicates clearly that prospidin is quite chemically stable in comparison, e.g., with its analogues [5]. Therefore, it was possible to disregard its destruction in our case when the period of time between sampling of the papilloma and HPCE separation was not more than 2 h.

3.6. Determination of prospidin in clinical samples

To investigate the applicability of this method for the analyses of clinical samples, the amount of free prospidin in freshly cut papilloma of three patients who had been under drug treatment was determined. All sample preparation was similar to that during the calibration of analyses.

Table 2
Precision of the prospidin analysis

Concentration (μg/ml)	Retention time		Peak area	
	Intra-assay C.V. (%)	Inter-assay C.V. (%)	Intra-assay C.V. (%)	Inter-assay C.V. (%)
50	1.8	2.0	2.8	3.0
30	2.1	2.4	2.8	3.1
20	2.2	2.3	3.0	3.4
10	2.8	3.1	3.5	3.8
5	2.7	3.0	3.2	3.6

$n = 5$ for intra-assay; $n = 4$ for inter-assay.

On the resultant electropherograms (similar to Fig. 4) three main peaks were observed which were identified as disubstituted prospidin (peak N1, $t = 9.2$ min), an unknown compound which had also been found in calibrated samples (peak N2, $t = 11.4$ min) and DDTC (peak N3, $t = 16.5$ min). The correctness of this identification was confirmed by the addition of derivatized prospidin solution to the analyzed sample. After this treatment new peaks did not appear in the electropherograms, peak N1 increased and the other peaks diminished without changing the retention times.

Thus, peak N1 was identified as disubstituted prospidin. Its amount in three clinical samples according to calibration curve data was determined as 0.57, 0.87 and 1.43 mg/ml homogenate. Intra-day C.V. ($n = 4$ for every clinical sample) was found to be 0.8%, 0.8% and 1.0%, respectively. This allows us to conclude that using this advanced method of prospidin introduction, its amount in human tissue is more than the necessary minimum therapeutical level [2].

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