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USE OF REVERSED-PHASE C_{18} SEP-PAK CARTRIDGES FOR THE PURIFICATION AND CONCENTRATION OF SEPIAPTERIN AND OTHER PTERIDINES

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SUMMARY

Several pteridines have been tested for their ability to bind to C_{18} Sep-Pak. Riboflavin, sepiapterin, deoxysepiapterin, 6-acetyl-7,8-dihydropterin, 6-acetyldihydrohomopterin and 3'-hydroxysepiapterin were strongly retained and all but 6-acetyldihydrohomopterin quantitatively recovered upon elution with 2 ml of methanol. The effects of the concentration and volume of the sample, pH and salt concentration on the retention of sepiapterin have been studied. The procedure was very useful for the purification, desalting, solvent exchange and concentration of pteridines having high affinity for the cartridge. C_{18} Sep-Pak has been applied successfully to sample clean-up prior to high-performance liquid chromatography, and to the assay of the dihydropterin oxidase and biopterin synthase activities of *Drosophila melanogaster*.

INTRODUCTION

Pteridines are a family of biologically important compounds to which belong riboflavin and folic acid. In recent years, it has been found that other less known members of this family, such as neopterin, biopterin and sepiapterin, are widespread and some of their derivatives play an important role in the metabolism of aromatic amino acids[1–5]. It is now well established that a blockage in the biosynthesis of 5,6,7,8-tetrahydrobiopterin leads to a type of severe phenylketonuria in humans[4,6–8], and the relationship between the abnormal excretion pattern of pteridines in urine and several other diseases has been demonstrated[9–13].

Fluorescence is exhibited by oxidized pteridines and their 7,8-dihydro derivatives, thus many quantitative analyses are based on their fluorometric detection[14–18]. However, since pteridines are normally found in very low concentrations in biological fluids, and sometimes in complex mixtures with other fluorescent metabolites and quenchers, a simple procedure for their purification and concentration is highly desirable.

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Octadecylsilane cartridges are increasingly being used for sample clean-up before chromatographic analyses[19–22] and for enrichment of trace compounds[23,24]. In the present work, the possibility of using C_{18} Sep-Pak cartridges for the purification and concentration of pteridines has been investigated. Applications to the prepurification of synthetic pteridines prior to high-performance liquid chromatography (HPLC) and to the measurement of dihydropterin oxidase and biopterin synthase activities are also described.

EXPERIMENTAL

Chemicals

C_{18} Sep-Pak cartridges were obtained from Waters Assoc. and Sephadex G-25 from Pharmacia. For the Sep-Pak procedure, analytical grade methanol and water distilled from glass apparatus were used.

Neopterin, 7,8-dihydroneopterin, 7,8-dihydrobiopterin, 6-formylpterin and 3'-hydroxysepiapterin were purchased from Dr. Schircks laboratory. Deoxysepiapterin (isosepiapterin) was a generous gift from Dr. W. Pfleiderer, 6-acetyl-7,8-dihydropterin from Dr. M. Tsusue and neopterin 3'-monophosphate from Dr. T. Shiota. 6-Acetyldihydrohomopterin was obtained from Drosophila heads as described earlier[25]. 7,8-Dihydroneopterin 3'-triphosphate was synthesized enzymatically from GTP using GTP cyclohydrolase I from *Escherichia coli* as described elsewhere[22]. The isolation of sepiapterin from Drosophila heads is described below. All the other pteridines were obtained from Sigma.

Sep-Pak procedure

The C_{18} Sep-Pak cartridges were attached to a Luer-tipped glass syringe. Prior to use, they were activated with 2 ml of methanol and 5 ml of water. The sample application, washing and elution were carried out at a flow-rate not exceeding 10 ml/min. In order to avoid photooxidation of pteridines, all operations were conducted in dim light. Except as indicated otherwise, the following protocol was used: 10 ml of sample were applied, the cartridge was washed with 3 ml of water and then eluted with 2 ml of methanol. Water was added to the washing and elution fractions to make the final volume of each 10 ml. The absorbances of the initial solution and of the three chromatographic fractions were measured at 420 nm in a Cecil CE 393 spectrophotometer. After each use, the cartridge was equilibrated with 3 ml of water.

For the experiment shown in Table I, this protocol was modified as follows: 2 ml of sample were used, the cartridge was washed with 2 ml of water and then eluted with 2 ml of methanol. The cartridge was equilibrated with 2 ml of water prior to the next experiment. The absorbance was measured at the wavelength of the absorption maximum for each pterin.

Preparation of sepiapterin

Sepiapterin was prepared from flies of the sepia vermilion double mutant of *Drosophila melanogaster*. All steps were performed under dim red light to avoid photooxidation of sepiapterin. A 50-g amount of adult flies (0–2 days old) were homogenized in 250 ml of 0.1 *M* hydrochloric acid in an electric blender. After the addition of 80 ml of 10% perchloric acid, the homogenate was centrifuged at 14 000

g for 20 min. The supernatant was neutralized with potassium hydroxide and the precipitate separated by decantation. The solution was concentrated to a final volume of 25 ml in a rotary evaporator at 45°C (after concentration the pH was 6.96). After centrifugation at 14 000 *g* for 10 min, the supernatant solution was applied to a Sephadex G-25 column (fine, 73 × 2.7 cm) which had previously been washed with 100 ml of 0.1 *M* hydrochloric acid and then with water until pH > 6. Elution was carried out with water at a flow-rate of 2 ml/min and 7-ml fractions were collected. Sepiapterin was eluted free from other pteridines between fractions 81 and 94. The combined fractions were kept at −20°C in the dark until required. Around 5.5 mg of pure sepiapterin were obtained assuming an extinction coefficient of 10 400 M^{-1} cm^{-1} at 420 nm in water.

Preparation of Drosophila extracts for enzyme assays

For the preparation of the crude extract the method of Fan and Brown[26] was followed. Fly heads (0.925 g) of the purple cardinal double mutant of *Drosophila melanogaster* were mixed with 4 ml of 0.1 *M* PIPES buffer, pH 7.5, containing 10 m*M* 2-mercaptoethanol. The suspension was homogenized in a glass homogenizer and centrifuged at 20 000 *g* for 45 min. The clear supernatant is the "crude extract" and was kept at −20°C in 10% glycerol prior to use.

To obtain a pteridine-free extract, the procedure of Neal and Florini[27] was used: aliquots of the "crude extract" (each 0.4 ml) were applied to Sephadex G-25 columns (5.5 × 1 cm) equilibrated with 50 m*M* Tris–HCl buffer, pH 7.5, and centrifuged at 150 *g* for 1 min. Tris–HCl buffer (0.4 ml) was added to the columns and the centrifugation was repeated to elute the protein fraction remaining in the columns. The chromatographic fractions were combined and used for the enzyme assays.

Enzyme assays

The conditions for the assays were as given by Fan and Brown[26]. The reaction mixture for the assay of dihydropterin oxidase activity contained (in a final volume of 0.510 ml) 33 m*M* Tris–HCl (pH 7.5), 14.5 μM sepiapterin and enzyme extract (1.1 mg of protein). The reaction mixture for the assay of biopterin synthase activity contained in addition 2 m*M* β-nicotinamide–adenine dinucleotide phosphate, reduced form (NADPH). A blank with boiled enzyme extract was prepared containing Tris–HCl, sepiapterin and NADPH. Incubation was carried out at 30°C in the dark. The fluorescence was measured in a Perkin-Elmer Model MPF-44B fluorescence spectrophotometer (excitation at 420 nm, emission at 520 nm, both slits of 10 nm). The protein determination was carried out according to Bradford[28].

Reversed-phase chromatography of synthetic pteridines

Chromatography was carried out with 12% methanol in water in a column (30 × 0.4 cm) of C_{18} μBondapak from Waters Assoc. kept at 35°C. The flow-rate was maintained at 1 ml/min with a pressure of 1500 p.s.i. using a Waters 6000A pump. The fluorescence was monitored with a Schoeffel FS 970 fluorescence monitor set at 360 nm for excitation and a 418-nm cut-off emission filter.

RESULTS

Retention of pteridines in C_{18} Sep-Pak

The capacity of C_{18} Sep-Pak to retain a variety of synthetic and natural pteridines was tested and the results are shown in Table I. Only a few pteridines were quantitatively retained under the assay conditions. Pteridines with retention percentages lower than 70% would be eluted from the cartridge if higher volumes of water were passed through. We have successfully used C_{18} Sep-Pak cartridges for the routine separation of riboflavin from isoxanthopterin after Sephadex fractionation of Drosophila pteridines as described in the Experimental section (data not shown). Among the pteridines strongly retained, only 6-acetyldihydrohomopterin was not quantitatively eluted with 2 ml of methanol. Riboflavin, sepiapterin, deoxysepiapterin, 6-acetyl-7,8-dihydropterin and 3'-hydroxysepiapterin, which show a strong yellow fluorescence when bound to the cartridge, left no fluorescence after elution with 2 ml of methanol.

Effects of sample concentration and volume on the retention of sepiapterin

Aqueous solutions of sepiapterin were used to study the influence of the volume

TABLE I

SELECTIVITY OF C_{18} SEP-PAK IN THE RETENTION OF PTERIDINES

Pteridines were dissolved in water, applied to the cartridge, washed with water and eluted with methanol. The absorbance of the initial sample and of the flow-through fractions was measured. Since the extinction coefficients in methanol are slightly different from those in water for most pteridines, only aqueous fractions were used to calculate the percentages of retention.

Pteridine	*Wavelength used (nm)*	*Absorbance*				*Retention (%)*
		Initial sample	*Sample through*	*Washing fraction*	*Methanolic fraction*	
Riboflavin	440	0.247	0	0	0.252	100
Deoxysepiapterin	420	0.197	0	0	0.208	100
Sepiapterin	420	0.451	0	0	0.479	100
6-Acetyl-7,8,-dihydropterin	420	0.206	0	0	0.222	100
6-Acetyldihydrohomopterin	383	0.547	0	0	0.469	100*
3'-Hydroxysepiapterin	420	0.367	0	0.024	0.359	93
Biopterin	345	0.343	0.028	0.075	0.222	70
6-Formylpterin	345	0.071	0.010	0.014	0.035	66
6-Hydroxymethylpterin	345	0.371	0.018	0.134	0.200	59
7,8-Dihydrobiopterin	316	0.512	0.027	0.209	0.268	54
7,8-Dihydroneopterin	316	0.610	0.041	0.244	0.332	53
Isoxanthopterin	333	0.112	0.006	0.055	0.044	46
Pterin	345	0.240	0.040	0.097	0.125	43
Leucopterin	336	0.097	0.026	0.046	0.013	26
Xanthopterin	387	0.120	0.030	0.077	0.021	11
Folic acid	345	0.108	0.051	0.048	0.017	8
Neopterin	345	0.320	0.115	0.178	0.026	8
Neopterin 3'-phosphate	345	0.444	0.337	0.088	0.011	4
6-Pterincarboxylic acid	345	0.183	0.144	0.041	0.002	0

* Two further elutions with 2 ml of methanol each gave solutions with absorbances of 0.025 and 0.006 respectively.

TABLE II

EFFECT OF THE CONCENTRATION AND VOLUME OF SAMPLE ON THE RETENTION OF SEPIAPTERIN

Sepiapterin dissolved in water was applied to the cartridge, washed with 3 ml of water and eluted with 2 ml of methanol. The final volume of all chromatographic fractions was adjusted to 10 ml with water. Retention percentages for 10-ml samples were calculated by comparing the absorbances of the methanolic fractions with those of the initial samples. For 5- and 2.5-ml samples the retention was calculated by comparing the absorbances of the chromatographic fractions.

Sample number	*Sepiapterin concentration (μM)*	*Sample volume (ml)*	*Absorbance at 420 nm*				*Retention (%)*
			Initial sample	*Sample through*	*Washing fraction*	*Methanolic fraction*	
1	4.6	10	0.048	0.001	0.001	0.048	100
2	11.3	10	0.118	0.001	0.002	0.117	99
3	23.1	10	0.240	0.003	0.007	0.226	94
4	44.4	10	0.462	0.012	0.017	0.434	94
5	90.1	10	0.943	0.047	0.060	0.836	89
6	45.6	5	0.474	0.001	0.003	0.231	98
7	88.1	5	0.916	0.003	0.008	0.456	98
8	86.0	2.5	0.894	0.000	0.001	0.234	100

and concentration of the sample on the performance of the cartridge. The results presented in Table II indicate the good capacity of the cartridge for sepiapterin: 89% of the sepiapterin from 10 ml of a 90.1 μM solution is retained and recovered upon elution. When different volumes of sample containing the same amount of sepiapterin are applied to the cartridge, the higher the concentration the better is the recovery

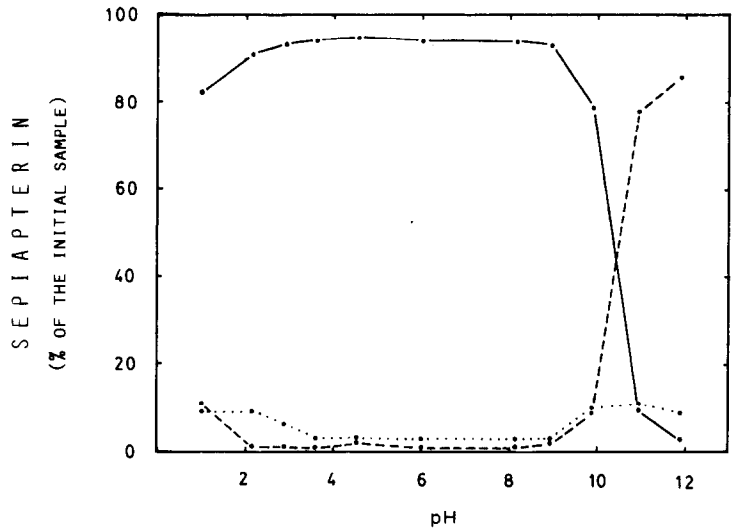

Fig. 1. Influence of the pH on the retention of sepiapterin on C_{18} Sep-Pak. Samples (10 ml) of 11.3 μM sepiapterin in water at different pH values were used. After application of the sample, the cartridge was washed with 3 ml of water and then with 2 ml of methanol. – – –, Sepiapterin not retained; ....., sepiapterin eluted with water; ———, sepiapterin eluted with methanol.

TABLE III

EFFECT OF SALT CONCENTRATION ON THE RETENTION OF SEPIAPTERIN

Samples (10 ml) of 11.3 μM sepiapterin in water containing different concentrations of salt were used. The sepiapterin retention was calculated as the percentage of the initial sepiapterin recovered in the methanolic fraction.

Type of salt	*Concentration (M)*	*Sepiapterin retention (%)*
Sodium acetate, pH 5.5	0.1	94
	0.5	93
	1.0	91
Potassium phosphate, pH 7.5	0.1	95
	0.5	93
	1.0	92
Tris–HCl, pH 8.5	0.1	95
	0.5	94
	1.0	88
	2.0	77
Potassium chloride	0.5	93
	1.0	87
	2.0	84
	3.0	76

of sepiapterin in the methanolic fraction. This is seen in Table II when samples 3, 6 and 8 (containing the same amount of sepiapterin), or samples 4 and 7, are compared.

Effect of pH on the retention of sepiapterin

The retention of sepiapterin on C_{18} Sep-Pak was tested in the range pH 1.0–11.9. The results shown in Fig. 1 are in agreement with those expected considering the dissociation constants of sepiapterin. This pteridine has a pK_a value of 1.27 for the equilibrium of the protonated and neutral molecules and of 9.95 for the equilibrium of the neutral and anionic molecules[29]. Recoveries around 94% were obtained in the pH range where sepiapterin exists as a neutral molecule. The retention decreased markedly as the pH was increased or decreased to values where sepiapterin is mainly present in the ionized form.

Effect of salt concentration on the retention of sepiapterin

Several concentrations of common buffers and of potassium chloride were tested. The results (Table III) show that concentrations up to 0.5 *M* hardly interfere with the sepiapterin retention. Higher salt concentrations gradually diminish the ability of sepiapterin to bind to the cartridge.

Application to the analysis of dihydropterin oxidase and biopterin synthase activities of Drosophila melanogaster

The dihydropterin oxidase and biopterin synthase activities were assayed in a pteridine-free extract of *D. melanogaster* as described in the Experimental section. The results are shown in Fig. 2 and the Sep-Pak procedure is described in the caption.

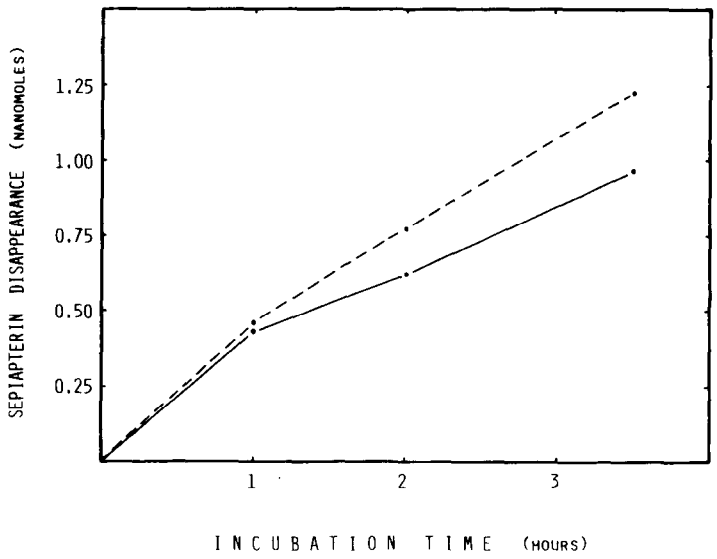

Fig. 2. Assay of the dihydropterin oxidase and biopterin synthase activities in *Drosophila melanogaster*. Aliquots (150 μl) of the reaction mixture were taken at different times and boiled for 1 min. The precipitated proteins were separated by centrifugation at 10 000 *g* for 2 min. Each supernatant was applied to a C_{18} Sep-Pak cartridge together with 1 ml of water used to rinse the reaction tube. The cartridge was washed with 2 ml of water. The eluted sample and washing fractions were discarded. The retained sepiapterin was eluted with 2 ml of methanol and its fluorescence measured. ———, Reaction mixture without NADPH; – – –, reaction mixture with NADPH.

The purification step with Sep-Pak eliminated the contribution of NADPH to the fluorescence of sepiapterin and most of the fluorescentcontaminants present in the enzyme extract.

The dihydropterin oxidase activity was estimated from the decrease of sepiapterin in the reaction mixture containing no NADPH. The blank with boiled enzyme exhibited no sepiapterin degradation. The biopterin synthase activity was measured, together with the dihydropterin oxidase activity, in the reaction mixture containing NADPH. Since dihydropterin oxidase only requires molecular oxygen to work, enzymatic oxidation of sepiapterin is taking place simultaneously in the reaction mixture for the assay of biopterin synthase.

Application to the purification of synthetic pteridines before HPLC analysis

When 7,8-dihydroneopterin 3'-triphosphate is heated at 100°C in the presence of Tris–HCl pH 8.5, a number of pteridine derivatives are formed in low yield: X, X1, X2[30] and X3. By use of C_{18} Sep-Pak cartridges, the residual dihydroneopterin triphosphate and its main degradation products (neopterin triphosphate, dihydroneopterin and neopterin di- and monophosphates, dihydroneopterin, neopterin and 6-pterincarboxylic acid) are separated from the compounds of interest since the former are too hydrophilic to be retained. As these degradation products are the predominant components of the boiling reaction mixture, when the Sep-Pak step is omitted they interfere in the subsequent chromatographic separation, partially overlapping with X1 and completely masking X3. Fig. 3 shows two chromatograms from

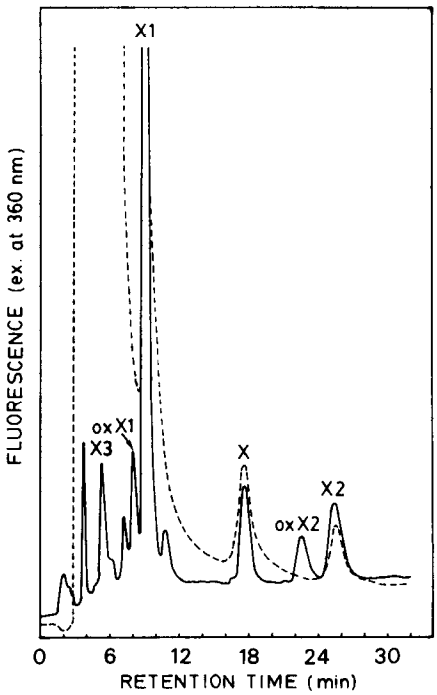

Fig. 3. Separation of 7,8-dihydroneopterin 3'-triphosphate derivatives (X, X1, oxidized X1, X2, oxidized X2 and X3). Dihydroneopterin triphosphate was synthesized enzymatically using GTP cyclohydrolase I in 1-ml reaction mixtures containing 0.33 m*M* GTP, 0.1 *M* Tris, 0.1 *M* sodium chloride and 0.01 *M* EDTA (pH 8.5). After incubation for 24 h at 42°C in the dark, the reaction tubes were heated at 100°C for 90 min. The precipitated proteins were separated by centrifugation at 10 000 *g* for 4 min. The supernatants were either applied directly for HPLC or passed through the C_{18} cartridge, washed with 2 ml of water and eluted with 1 ml of methanol; methanol was removed in a rotary evaporator at 40°C and the dry sample dissolved in water before HPLC analysis. ———, Sample after Sep-Pak clean-up; – – –, untreated sample.

two different reaction mixtures obtained with and without the use of C_{18} Sep-Pak in the preparation of the sample.

DISCUSSION

Several pteridines have been tested for their ability to bind to C_{18} Sep-Pak when dissolved in aqueous solutions. A wide range of adsorptivities has been found among the pteridines tested. Some pteridines bind strongly to the cartridge (100% of retention) and others are hardly retarded (0–11% of retention) (Table I). These differences in adsorptivity are mainly due to the ionizable groups and the number and type of hydrophilic groups present in the molecule. The only compound that was not quantitatively eluted using 2 ml of methanol was 6-acetyldihydrohomopterin. Though metabolically related to pteridines, this compound is not a pteridine but a pyrimidodiazepine (it has a seven- and a six-membered ring instead of two six-membered rings as in the pteridines). Its anomalous behaviour in reversed-phase chro-

matography and its differences from pteridines have already been pointed out[31].

The Sep-Pak procedure can be applied to the purification of pteridines in two ways: (1) in the case of pteridines with intermediate or low affinity for the cartridge, to eliminate hydrophobic contaminants present in the sample, and (2) in the case of pteridines with high affinity, to eliminate hydrophilic contaminants, to exchange the solvent and to desalt and concentrate the sample.

Sepiapterin has been chosen to determine the parameters pertaining to the performance of the cartridge because of its biological importance, because it is one of the pteridines that strongly bind to the cartridge and because its binding is weak enough to allow a small fraction of sepiapterin to be eluted when rigorous conditions are used. The results show that the sepiapterin recovery depends on the concentration and volume of the sample. Both variables are related since the use of lower sample volumes allows a quantitative recovery in more concentrated samples (Table II). The pH of the solution can also affect pteridine retention by resulting in ionization of groups in the molecules. In the case of sepiapterin a wide range of pH can be used with no effect on its retention (Fig. 1). The salt concentration also affects sepiapterin retention but only when high concentrations of salt are used (Table III). The concentrations normally employed in biological buffers do not affect the retention.

Other organic solvents, such as ethanol, isopropanol and acetonitrile, could be used to elute pteridines from the cartridge, but methanol was preferred because of its low boiling point, low toxicity and good pteridine solubility. After elution of the sample from the cartridge, the methanol can be removed either by gently heating at reduced pressure or by use of a stream of nitrogen at room temperature.

The same cartridge can be used several times without noticeable loss of efficiency. We have used a cartridge 25 times with solutions of sepiapterin and have seen no loss in the retention capacity. For qualitative work, for example when sepiapterin, deoxysepiapterin or riboflavin is purified and concentrated from Drosophila extracts, a single cartridge can be used almost indefinitely.

The application of C_{18} Sep-Pak to the measurement of the dihydropterin oxidase and biopterin synthase activities illustrates its advantages in pteridine separation and purification. In our procedure, the substrate consumption was measured in order to estimate the enzyme activities, and C_{18} Sep Pak was used to separate sepiapterin from other fluorescent contaminants that interfered with the assay. Other assay procedures can be used in which measurement of the product formed, instead of the sepiapterin consumed, is employed. This is advisable when low reaction yields are obtained. In the case of radioactive sepiapterin as substrate, the conditions of the assay (pH, salt concentration or addition of methanol to the washing water) could be modified so as to achieve a complete separation of sepiapterin from oxidized sepiapterin or dihydrobiopterin. Another application of C_{18} Sep-Pak is in the clean-up of samples before HPLC analysis. Its use gave rise to the discovery of X3, a new member of the X family that had been masked by the simultaneous elution with other contaminants when the Sep-Pak step was omitted. In addition, the reaction products could be concentrated from samples of 20 ml to 0.5 ml by a single Sep-Pak step.

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