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Note

Analytical recovery of protein-bound pyridoxal-5′-phosphate in plasma analysis

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Pyridoxal-5′-phosphate (PLP) exists largely as a Schiff-base-linked complex to ε-amino residues of albumin in plasma [1—3]. At least two high-affinity PLP binding sites on albumin and numerous binding sites with lower affinity have been described [1] Most methods employed in plasma PLP determinations require trichloroacetic acid (TCA) precipitation of plasma proteins [4—6] Although PLP added to plasma is in general satisfactorily recovered, reliability of this procedure was questionable since PLP coupling to albumin is presumably time-dependent In one case, spiking of plasma with PLP was done after protein precipitation, which yields no information on possible protein retention of PLP [7] At least one report states that TCA precipitation does not liberate PLP completely from albumin and pre-incubation with semicarbazide is suggested to free albumin-linked PLP [8] We therefore investigated the possibility that TCA plasma protein precipitation does not liberate albumin-bound PLP quantitatively

We present evidence that TCA precipitation is fully sufficient to liberate PLP from albumin In addition, we demonstrate that semicarbazone derivatisation of PLP and pyridoxal (PL) proceeds quantitatively in presence of 3 3% TCA. Therefore, a previously described method for plasma PLP and PL analysis based on pre-column semicarbazone derivatisation [9] is simplified by simultaneous addition of TCA and semicarbazide to plasma. This improvement renders the technique even more suitable for large-scale population screening studies of vitamin B_6 status

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EXPERIMENTAL

Reagents

PL and PLP were obtained from Merck (Darmstadt, F R.G.) and purified as described previously [9]. [4,5-^{14}C]PLP (1.93 mCi/mmol) was supplied by Amersham (Buckinghamshire, U.K.). The internal standard, 6-methyl-2-pyridine carboxaldehyde semicarbazone (MPCSC), was prepared from 6-methyl-2-pyridine carboxaldehyde (Aldrich, Milwaukee, WI, U.S.A.) as described previously [9]. Bovine albumin (Fraction V) was bought from Sigma (St Louis, MO, U S A)

PLP binding to albumin

[4,5-^{14}C]PLP was added to a 3% bovine albumin solution in 0 05 *M* phosphate buffer, pH 7.2, containing 0.154 *M* sodium chloride (phosphate-buffered saline, PBS) to a final concentration of 6 4 μg/ml PLP in albumin solution The mixture was incubated for 90 min at 37°C and subsequently a 0.5-ml aliquot was applied to a 30 × 2.2 cm Sephadex G-50 (Pharmacia, Uppsala, Sweden) column PBS was used to elute the column at a flow-rate of 40 ml/h, and the eluate was fractionated at 4-min intervals for 90 min A control experiment was run with PLP added to PBS without albumin.

A 0 5-ml aliquot of each fraction was withdrawn for ^{14}C measurement. Triplicate 0.5-ml aliquots were withdrawn for albumin precipitation using 0 25 ml of 10% TCA. The clear supernatant obtained after centrifugation (25 000 *g*, 15 min) was quantitatively recovered for radioactivity determinations Albumin precipitates were washed twice with 10% TCA and the supernatants quantitatively recovered for ^{14}C measurement. Finally, the precipitated albumin was suspended in 0.5 ml PBS and used for ^{14}C measurement

Reaction of PLP and PL with semicarbazide under acid conditions

Aliquots (1 ml) of a PLP or PL solution (10 μg/ml) were incubated at 37° C for 30 min in the presence of 0 5 ml of 10% TCA and either 0 3 ml of 0 5 *M* semicarbazide or 0.3 ml water, after washing with diethyl ether and dichloromethane, 30 μl of each water phase were injected onto a Whatman (Clifton, NJ, U S A) Partisil-10 ODS-3 column (25 cm × 4.6 mm I D , 10 μm particle size) using a mobile phase as described previously [9] The column eluate was monitored by UV detection at 290 nm.

PLP and PL recoveries from albumin complexes or plasma

Aliquots (1 ml) of a 3% albumin solution were spiked with 5.0 ng PLP and 2 05 ng PL, 10 0 ng PLP and 4.10 ng PL and 20 0 ng PLP and 8.20 ng PL, respectively, and incubated for 90 min at 37° C This procedure was repeated with 1-ml aliquots of plasma, containing a sodium phosphate buffer (pH 7 2) at a final concentration of 0 02 *M* PLP and PL concentrations in each aliquot were determined using a modification of a previously described high-performance liquid chromatographic (HPLC) technique [9] Briefly, to 1 0 ml of albumin solution or plasma, 0 5 ml of 10% TCA, 0 3 ml of 0 5 *M* semicarbazide and 50 μl MPCSC ($8.2 \cdot 10^{-4}$ *M*) were simultaneously added The mixture was mixed vigorously and then heated at

40° C for 30 min. The clear supernatant obtained after centrifugation was washed twice with diethyl ether and once with dichloromethane. A 30-μl aliquot of the water phase was analysed for PLP and PL using HPLC as described previously [9] except that a Whatman Partisil-5 ODS-3 RAC II analytical column (10 cm × 4 6 mm I.D , 5 μm particle size) was used

RESULTS AND DISCUSSION

Fig. 1 indicates that the Sephadex G-50 column used was very effective in the separation of PLP and albumin. When albumin was pre-incubated in presence of [^{14}C]PLP, 85% of radioactivity was associated with the albumin peak and 15% PLP eluted in the free position Although the PLP concentration used was 450-fold higher than the mean plasma PLP level of 14.3 ng/ml in adult, healthy men [10], it can be calculated that only 2 9% of the high affinity binding capacity of albumin would be occupied by the added PLP, assuming a molecular weight of 67 000 for albumin, two high-affinity PLP binding sites per albumin molecule [1] and 100% binding of PLP to albumin The distribution of radioactivity following protein precipitation and resolubilisation of albumin-containing fractions indicates that [4,5-^{14}C]PLP was practically quantitatively liberated from albumin by a final TCA concentration of 3 3% (Table I) The final TCA concentration (3 3%) used was lower than those reported by others in vitamin B_6 analysis [4–6], however, a 3 3% final TCA concentration was reported to precipitate more than 99% of plasma proteins [11]

Generally the pH optimum for the semicarbazone reaction is slightly acidic [12, 13], but Fig 2 illustrates that PLP and PL react quantitatively to form the respective semicarbazones in the presence of excess semicarbazide in 3 3% TCA (pH 2 0 [11]) Using reversed-phase HPLC, underivatized PLP and PL eluted both after 4 0 min, while the semicarbazone derivatives of PLP and PL

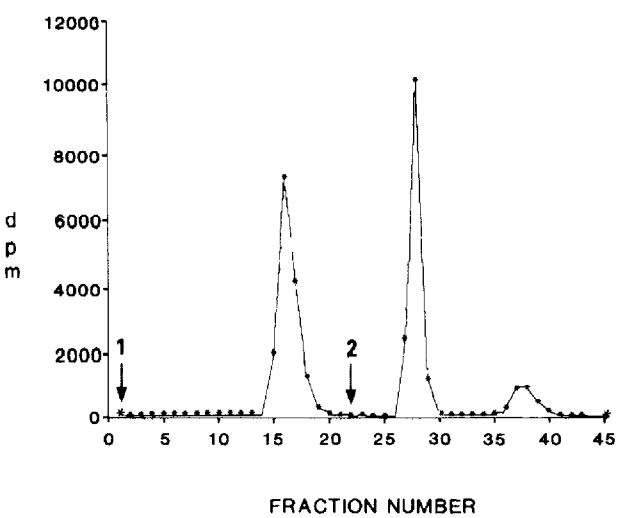

Fig 1 Binding of [4,5-^{14}C]PLP to albumin [4,5-^{14}C]PLP was incubated in PBS (blank solution) or in PBS containing 3% albumin Blank solution was applied at 1, albumin containing solution at 2

TABLE I

RECOVERY OF [4,5-^{14}C]PLP FROM ALBUMIN COMPLEX

Fractions 28 and 29 (Fig 1) (identified as containing albumin) were treated as described under Experimental and the distribution of radioactivity in the supernatant and precipitate established by ^{14}C measurement. Radioactivity (%) recovered in supernatant is calculated as the sum of radioacitivity (%) measurements in supernatant and was solutions

Fraction No	Whole fraction (dpm)	Super-natant (dpm)	Wash solution (dpm)	Solubilised precipitate (dpm)	Radioactivity recovered in supernatant (%)	Radioactivity bound to protein (%)
28	10074	9961	448	92	103 3	0 9
29	1273	1252	47	12	102 0	0 9

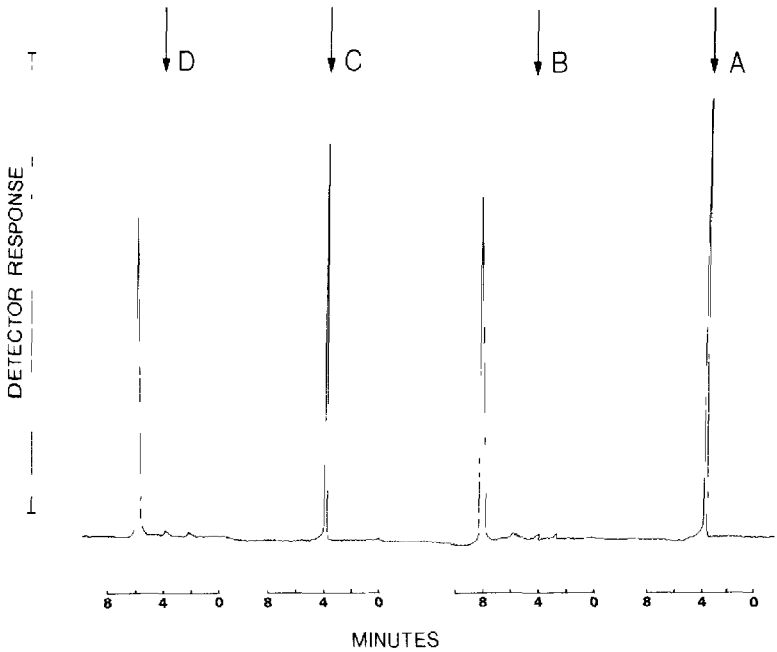

Fig 2 The reaction of PLP and PL with semicarbazide in 3 3% TCA HPLC and UV detection were used to determine whether semicarbazone derivatisation was quantitative (see text) (A) Purified PL, (B) PL reacted with semicarbazide, (C) purified PLP, (D) PLP reacted with semicarbazide Arrows indicate the position of the PL peak (A and B) or the PLP peak (C and D)

eluted at 5.8 and 7.8 min, respectively (Fig. 2) The amount of UV-absorbing material eluting at 4 0 min after semicarbazone derivatisation was negligible both in the case of PL and PLP, thus indicating that the semicarbazone derivatisation was complete under the reaction conditions used Applying this to plasma PLP and PL analysis it may be concluded that it is unnecessary to adjust the pH of the TCA supernatant in order to perform semicarbazone derivatisation as reported by others [14] Therefore, our previously reported sample preparation procedure [9] for HPLC has been modified by adding TCA and semicarbazide simultaneously to plasma (see Experimental)

TABLE II

RECOVERY OF PLP AND PL ADDED TO A 3% ALBUMIN SOLUTION OR PHOSPHATE-BUFFERED PLASMA

Added (ng/ml)	Found (mean ± S D , n = 5) (ng/ml)		Recovery (%)	
	3% Albumin	Plasma	3% Albumin	Plasma
PLP				
0	15 47 ± 0 49	26 01 ± 0 49	—	—
5	20 06 ± 1 48	30 77 ± 1 04	91 8	95 2
10	25 24 ± 1 05	35 32 ± 1 46	97 7	93 1
20	34 14 ± 1 71	46 82 ± 2 13	93 4	104 1
PL				
0	3 61 ± 0 08	12 91 ± 0 71	—	—
2 05	5 56 ± 0 13	15 09 ± 0 73	95 1	106 3
4 10	7 91 ± 0 22	17 15 ± 0 75	104 9	103 4
8 20	12 16 ± 1 01	21 54 ± 0 80	104 3	105 2

Analysis of albumin solution aliquots pre-incubated with small PLP and PL spikes to allow PLP binding to albumin yielded excellent recoveries for both PLP and PL (Table II) Using a short (10 mm × 4 6 mm I.D) high-resolution reversed-phase column, good resolution was obtained between MPCSC and the semicarbazone derivatives of PLP and PL (Fig 3) Note that the separation was completed within 6 min in comparison to the 11 min required [9] when a Partisil-10 ODS-3 analytical column (25 cm × 4.6 mm) was used. It is of

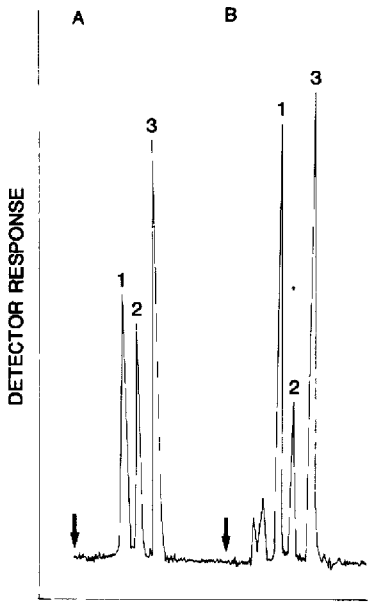

Fig 3 Determination of PLP and PL (A) Standard containing 12 0 ng/ml PLP and 5 0 ng/ml PL, (B) plasma sample (17 4 ng/ml PLP and 3 0 ng/ml PL) Arrows indicate when the sample was injected Peaks 1 = PLP semicarbazone (2 50 min), 2 = PL semicarbazone (3 24 min), 3 = MPCSC, the internal standard (4 28 min)

interest to note that commercial albumin contained PLP (and sometimes even PL) indicating that the Schiff-base-linked PLP is not removed from albumin during the usual protein isolation steps However, the amount of PLP bound to the commercial albumin preparation was negligible when the potential PLP binding capacity of albumin was taken into account From Table II it can be calculated that only 0 007% of the high affinity binding capacity of albumin was occupied by PLP. It is therefore reasonable to accept that the endogenously bound PLP did not adversely affect spiked PLP binding to albumin in any way

To summarize, we present evidence that TCA precipitation of plasma proteins liberated albumin-bound PLP completely A previously reported HPLC method [9] for plasma PLP and PL determinations is improved by (a) doing plasma protein precipitation and semicarbazone derivatisation of PLP and PL simultaneously and (b) the use of a short high-resolution reversed-phase column thereby cutting HPLC analysis time by more than 40% per sample analysed

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