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Short communication

Nitritocobalamin and nitrosocobalamin may be confused with sulfitocobalamin using cation-exchange chromatography

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Abstract

The previously reported ability of SP-Sephadex C_{25} column chromatography for partitioning biologically important cobalamins has been modified to include analytical separation of nitritocobalamin (NO_2-Cbl) and nitrosocobalamin (NO-Cbl). Gel column dimensions (1.5 × 11.0 cm), a low eluent flow-rate (125 μl/min), collection of small eluate fractions (160 μl) plus maintenance of He saturated mobile and gel phases all combined to eliminate ordinarily confusing proximal elution of NO_2-Cbl and NO-Cbl with sulfitocobalamin (SO_3-Cbl) and cyanocobalamin. Cobalamin elution profiles from the gel column were monitored by direct radiometric analysis of ^{57}Co-labelled cobalamin standards or competitive intrinsic factor radioassays for cobalamin sample sizes up to 10.0 ng. Failure to implement the chromatographic conditions detailed here totally obscured analysis of NO_2-Cbl coexisting with SO_3-Cbl in brain tissues for chicks exposed to dietary sulfites and caused oversight of NO-Cbl normally coexisting in prepared NO_2-Cbl standards.

1. Introduction

Ion-exchange chromatography (IEC) using SP-Sephadex C_{25} permits analytical separation of commonly occurring forms of cobalamin (vitamin B_{12}) found in biological samples [1–7]. Cobalamins consecutively elute from the ion exchanger in the order of sulfitocobalamin (SO_3-Cbl), cyanocobalamin (CN-Cbl), methylcobalamin (Me-Cbl), deoxyadenosylcobalamin (Ado-Cbl) and hydroxocobalamin (OH-Cbl) [1,2]. Following separation and elution of these cobalamins from an IEC column, they can be detected using on-line UV detection, but only sensitive radioisotope dilution assays (RIDAs) of individual eluate fractions can quantitate picogram cobalamin amounts found in small tissue or fluid samples [8].

Combined application of the IEC–RIDA analytical approach for measuring SO_3-Cbl concentration levels in chick brains as part of a dietary sulfite study revealed a consistent post-elution asymmetry of the initial SO_3-Cbl elution peak in over 60 brains studied [2]. After preparing numerous cobalamin adduct species by established methods [9–12] plus the modification of IEC conditions from earlier applications [1,2], nitritocobalamin (NO_2-Cbl) [11] was identified

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as the cause of SO_3-Cbl asymmetry. Further studies also revealed that the modified IEC method reported here could resolve a nitrosocobalamin (NO-Cbl) reaction product present as a coexisting in vitro by-product of nitrite adduct formation with OH-Cbl [10] or alternatively, as a primary reaction product of nitric oxide (NO) with OH-Cbl. Neither the routine quantification of NO_2-Cbl in biological samples nor the detection of NO-Cbl generated in conjunction with NO_2-Cbl preparation have been reported in the literature. Moreover, any failure to partition NO_2-Cbl and NO-Cbl from SO_3-Cbl will produce overestimated concentrations of SO_3-Cbl using previously reported IEC methods and obscure any noteworthy analytical significance of NO_2-Cbl.

Paucities in the literature regarding NO_2-Cbl occurrence reflect its infrequent and anecdotal recognition in biological systems since its discovery in 1951 [11]. Based on the critical requirements for cobalamin in neurological systems, however, as well as recent verifications of enzyme mediated NO synthesis in the central nervous system, NO_2-Cbl occurrence may have more than anecdotal significance thereby necessitating its reliable quantification [12–15].

2. Experimental

2.1. Column preparation and operating conditions

Preparation of the SP-Sephadex C_{25} (Pharmacia, Piscataway, NJ, USA) gel was consistent with earlier reported methods [1] for construction of a 1.5×11.0 cm gel column. High purity deaerated water was saturated with He and supplied to the column at 125 μl/min. All polypropylene tubing serving column injector, inlet and eluent flows was 0.5 mm I.D.

2.2. Preparation of cobalamin chromatography standards

Cobalamin standards prepared for use over the column partitioning range of interest included CN-Cbl, SO_3-Cbl, NO_2-Cbl and NO-Cbl. All standards were prepared from CN^{57}CoCbl (Eastman Kodak Co., Rochester, NY, USA) following its conversion to OH^{57}CoCbl by established methods [10]. The conversion of OH^{57}CoCbl into SO_3^{57}CoCbl has been detailed by Kaczka et al. [10] and Smith et al. [11,12]. The in vitro preparation of NO^{57}CoCbl for use as a chromatographic standard required 500 μl of He saturated 0.05 *M* sodium acetate buffer at pH 5.20 containing any desired amount of radioactive tracer in the form of OH^{57}CoCbl. This reaction mixture was contained in a sealed 5.0 ml borosilicate reaction vial (21×62 mm) equipped with three gas flow access ports. One port provided a continuous supply of He sparged through the reaction mixture, another port supplied ebullient NO gas (Aldrich, Milwaukee, WI, USA) to the mixture, while the remaining port relieved gas pressure in the vial to a fume hood. Failure to eliminate oxygen from the reaction vessel readily converted the NO into nitrogen oxides [16] that gave large yields of NO_2^{57}CoCbl instead of the desired NO^{57}CoCbl product.

2.3. Cobalamin partitioning for standards and samples

Cobalamin separation performance of SP-Sephadex C_{25} gel columns was evaluated by applying individual ^{57}Co-labelled cobalamin calibration standards (800–6000 cpm) in a 200-μl volume of 0.05 *M* sodium acetate buffer at pH 5.20. Elution of the ^{57}Co-labelled cobalamin standards was monitored in 160-μl fractions using a Pharmacia LKB Clinigamma Twin System (Pharmacia LKB, Piscataway, NJ, USA).

To ensure underivatized transit of the NO^{57}CoCbl standard through the IEC column, the column was always preconditioned and run with He saturated water, and column injector ports were also placed under positive He pressure to minimize oxygen entry into the system. Moreover, all cobalamin standards and column chromatographic separations were conducted in the dark or under low red illumination.

Non-radioactive sample cobalamin extracts

from chick brains with total cobalamin concentrations up to 10.0 ng, determined by a conventional RIDA protocol (Becton Dickinson and Co., Orangeburg, NY, USA) were prepared for chromatographic study using hot ethanolic extraction [7], extract delipidation with *n*-hexane and final extract clean-up with 4.0 × 8.0 mm microcolumns of neutral activated alumina (Sigma, St. Louis, MO., USA) [17]. Relative concentration levels of tissue cobalamins separated over the IEC column were quantitated by RIDA of each 160-μl elution fraction collected. The linearity of RIDA logit plots used to quantitate cobalamins in each elution fraction over the range of 50–2000 pg/ml consistently demonstrated *r* values > 0.995. All IEC columns used for cobalamin analyses were used once and discarded to avoid run-to-run cross contamination of individual samples.

2.4. Animal test groups for brain cobalamin studies

Ten whole brains were randomly selected from each of three cohorts of Golden Comet cockerel hatchlings (Hall Brothers' Hatchery, North Brookfield, MA, USA) with average chick weights of 27.0 ± 2.5 g. Each cohort of 15 birds was individually housed in a separate heated brooder (Brower Co. Models 1680-2, Houghton, IA, USA) located in a 25°C laboratory with a 12 h light/dark cycle. One cohort served as control birds supplied with a sulfite-free basal ration while a second cohort was supplied with a basal ration plus 2.4% sulfite (w/w) as sodium sulfite. Basal ration specifications included corn meal (700.0 g), soy protein (300.0 g), sodium metasilicate (0.10 g) [18], *p*-aminobenzoic acid (0.11 g), sulfaguanidine (5.0 g) and a multivitamin–mineral mixture (70.0 g) [19] free from cobalamin and folate. A third chick cohort was given commercially available dehydrated instant potato granules found to contain a 187 ppm sulfite load according to the Monier–Williams method [20]. Food and water were provided ad lib. for 14 days to all birds. They were then sacrificed by ether anesthesia, whole brains were excised and then immediately stored frozen at −60°C until cobalamin extraction.

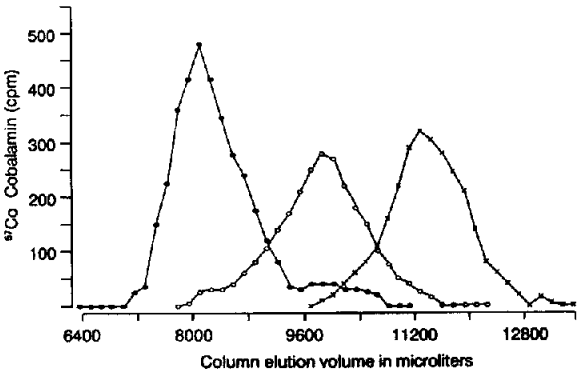

Fig. 1. Relative elution profiles for SO_3^{57}CoCbl (○), NO_2^{57}CoCbl (●) and CN^{57}CoCbl (×) standards separated on SP-Sephadex C_{25}.

3. Results and discussion

The relative elution profiles for cobalamin standards including SO_3^{57}CoCbl, NO_2^{57}CoCbl and CN^{57}CoCbl using chromatographic conditions reported above are shown in Fig. 1. Performance of the same method resolving NO_2-Cbl between SO_3-Cbl and CN-Cbl in cobalamin extracts of chick brains appears in Fig. 2. Elution profiles in Fig. 2 also demonstrate detectable differences in

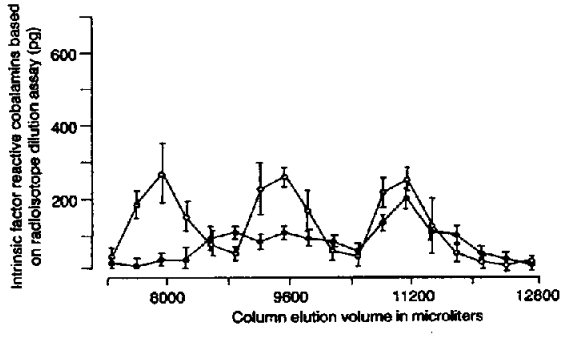

Fig. 2. Relative IEC elution profiles for RIDA detectable cobalamins (pg) in 14-day-old sulfite-free dietary control chick brains (●), compared to those in birds supplemented with 2.4% dietary sulfite (○). Points plotted represent cobalamins in eluate fractions (mean ± S.D.) for IEC analysis of ten individual brains in each dietary treatment group.

SO_3-Cbl appearance for birds supplied with sulfite-free control diets versus sulfite-containing rations as described in the previous section. Note that Fig. 1 plots cobalamin elution by direct radiometric monitoring of ^{57}Co-labelled standards while Fig. 2 relies upon intrinsic factor specific RIDAs for cobalamin quantification in each IEC column eluate fraction. Cobalamin forms coextracted with SO_3-Cbl, NO_2-Cbl and CN-Cbl including Me-Cbl, Ado-Cbl and OH-Cbl were not individually quantified since they were completely outside the scope of this animal study dealing with sulfite metabolism, but their separation concurred with previous studies [1,2].

The practical utility of the modified IEC method for quantification of SO_3-Cbl in the presence of NO_2-Cbl is shown in Table 1 for two cohorts of chicks supplied with two different sulfited rations and one cohort of sulfite-free control birds. The ng/g and relative percent occurrences for SO_3-Cbl, NO_2-Cbl and CN-Cbl with reference to total extractable brain cobalamins determined by RIDAs are tabulated. Total brain cobalamin amounts including quantitative contributions of Me-Cbl, Ado-Cbl and OH-Cbl showed 66.1 ± 3.1% decreases in both sulfite-supplemented rations. These notable cobalamin decreases occurred regardless of the large dietary differences in sulfite concentrations provided in chick feed rations as opposed to control birds. Exact analysis of variance significance (P) values for individual cobalamins present in sulfite supplemented birds versus control birds also appear in Table 1. A survey of individual chick brains (1.14 ± 0.12 g) spiked by injection of ^{57}Co-labelled cobalamin followed by extraction, IEC separation and radiometric analysis demonstrated a 9.6 ± 1.7% relative standard

Table 1
Quantification of sulfitocobalamin and nitritocobalamin relative to extractable brain cobalamins in two study cohorts of dietary sulfite supplemented chicks and a cohort of sulfite-free birds

Chick brain cobalamin concentration levels[a]	Sulfite-free diet[b] (control birds)	Sulfite-supplemented diet (2.4%, w/w)[b,f]	Sulfited, commercially prepared potato granules (187 ppm)[b,g]
Sulfitocobalamin			
concentration (%)	5.47 ± 5.11	26.54 ± 6.98 (P = 0.000)[c]	21.31 ± 7.63 (P = 0.004)[c]
(ng/g brain tissue)	0.30 ± 0.21	0.43 ± 0.30	0.43 ± 0.18
Nitritocobalamin			
concentration (%)	4.74 ± 2.83	19.14 ± 5.34 (P = 0.003)[c]	7.93 ± 5.09
(ng/g brain tissue)	0.26 ± 0.26	0.31 ± 0.31	0.16 ± 0.10
Cyanocobalamin			
concentration (%)	22.99 ± 12.90	17.78 ± 5.83	10.90 ± 6.46
(ng/g brain tissue)	1.26 ± 1.14	0.29 ± 0.27 (P = 0.093)[c]	0.22 ± 0.10 (P = 0.055)[c]
Total cobalamins[d]			
(ng/g brain tissue)	5.48 ± 2.59	1.62 ± 1.05 (P = 0.001)[c]	2.02 ± 0.347 (P = 0.008)[c]
Residual cobalamins[e]			
(ng/g brain tissue)	3.67 ± 2.04	0.59 ± 0.30 (P = 0.005)[c]	1.22 ± 0.33 (P = 0.015)[c]

[a] Independent analysis of ten birds per dietary study cohort.
[b] Mean ± 95% confidence interval.
[c] Analysis of variance for dietary sulfite cohort versus sulfite-free control based on Minitab 7.2 Vax/VMS statistical program.
[d] Total extractable cobalamins detected by RIDA.
[e] Combined total of methylcobalamin, deoxyadenosylcobalamin, hydroxocobalamin.
[f] Dietary protein 28%.
[g] Dietary protein 0.9%.

deviation (R.S.D.) for recoveries of the three respective cobalamin occurrences within a study cohort of ten birds. Run-to-run variations in precision for maximum peak elution of individual cobalamins at 8000, 9550 and 10 944 μl over ten replicate IEC separations was limited to a 1.42 ± 0.25% R.S.D. This R.S.D. also reflected the influence of new column construction for each of the ten IEC runs.

Inspection of Fig. 1 also reveals that SO_3^{57}CoCbl eluted at 8000 μl yet Fig. 2 shows evidence of another minor RIDA detectable cobalamin elution peak present at 8800 ± 100 μl for brain extracts of control birds. Although cobalamin elution in this region could not be discounted as SO_3-Cbl based on known literature [2], additional IEC using low sample loadings of NO_2^{57}CoCbl standard corroborated elution of an identical unknown minor cobalamin peak in the same region. Its occurrence was 19.5 ± 9.3% of the total radioactivity supplied for NO_2^{57}CoCbl standard preparation, quite unlike 93 + % yields offered by other preparations for SO_3^{57}CoCbl, CN^{57}CoCbl, or NO^{57}CoCbl. The persistent occurrence of this cobalamin at low concentrations after exposing OH^{57}CoCbl to nitrite anions at pH 5.20, and verification of its collateral appearance following OH^{57}CoCbl exposure to either N^{ω}-nitro-L-arginine or triglycerylnitrate in the distinct absence of sulfur compounds, all pointed to the common presence of a nitrogen oxide derivative of OH^{57}CoCbl.

Efforts to identify the unknown cobalamin eluting at 8800 ± 100 μl culminated in a reaction where OH^{57}CoCbl was exposed to 98.5% NO as outlined in the previous section. This rapidly produced 95 + % yields of NO ^{57}CoCbl product (Fig. 3) that unequivocally reproduced the elution behavior of the minor IEC peak. Furthermore, combined light irradiation and oxygen exposure of this NO^{57}CoCbl product gave a derivative consistent with the performance of NO_2^{57}CoCbl (Figs. 1 and 3). Substitution of sodium nitroprusside used in place of NO gas for reaction with OH^{57}CoCbl produced identical results (Fig. 4). Combined light and oxygen exposure beyond 24 h failed to produce markedly higher conversions of the NO^{57}CoCbl into

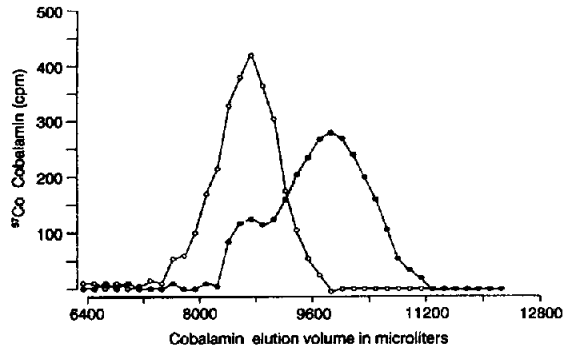

Fig. 3. Elution profile for NO^{57}CoCbl (○) resulting from OH^{57}CoCbl exposure to NO under oxygen free conditions in acetate buffer. See text for details. Exposure of this reaction product to an oxygen stream at 5.0 ml/min plus 500 W incandescent illumination distanced 75 cm from the sample for 30 min produced a cobalamin with IEC elution behavior identical to NO_2^{57}CoCbl (●). Note that a trace amount of the original NO^{57}CoCbl persists after oxygen exposure and illumination.

NO_2^{57}CoCbl than illustrated in Figs. 3 and 4. Beyond obvious combined light and oxygen effects illustrated, the relative occurrence of these cobalamins may depend upon pH conditions of the reaction environment. Previous studies by Stuehr and Nathan [21] support this contention since it has been shown that acid pH

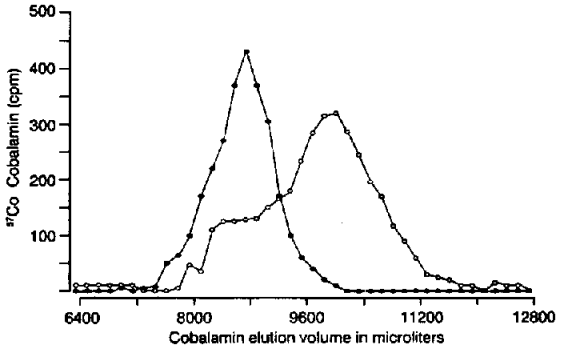

Fig. 4. Liberation of NO from nitroprusside followed by its reaction with OH^{57}CoCbl in acetate buffer produced a NO^{57}CoCbl product (●) with identical elution performance to that of purified NO gas reaction with OH^{57}CoCbl in Fig. 3. Exposure of the reaction product to the combined effects of light and oxygen similarly produced a cobalamin derivative with elution properties identical to NO_2^{57}CoCbl (○). As in Fig. 3, a trace of the original NO^{57}CoCbl continues to persist after its combined exposure to light and oxygen.

conditions surrounding the nitrite anion dictate the trace formation of nitrous acid ($pK_a = 3.4$) followed by its dismutation to yield reactive nitrogen intermediates (RNIs) including NO and nitrogen dioxide that could be reactive with OH-Cbl.

Although NO-Cbl and NO_2-Cbl have relatively low occurrences compared to other routinely studied brain cobalamins, the possibility exists that their detectable concentrations may only reflect a portion of their biogenically significant amounts since both cobalamins were found to decompose after 3.0 h in vitro incubation periods with 0.10 *M* ferrous ions or reduced glutathione.

4. Conclusions

Beyond the studies reported in this paper, the modified SP-Sephadex C_{25} IEC method has been useful for other analytical applications including the detection of NO_2-Cbl in the brain of the crab-eating monkey (*Macaca fascicularis*), detection of NO_2-Cbl secondary to *N*-methyl-D-aspartic acid potentiated NO synthetase activity in chick brain tissue, and detection of volatile RNIs in tobacco smoke upon reaction with OH-Cbl. Further application of the method may be useful for detecting clinically significant or biochemically important relationships involving NO and cobalamins apart from minimizing overestimation of SO_3-Cbl.

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