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Note

Rapid micromethod for the determination of Evan's blue in human plasma by high-performance liquid chromatography

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Evan's blue (T-1824) is an azo dye of unusual biomedical importance. Because of the high affinity of this compound for plasma albumin [1, 2], Evan's blue is used in the clinical determination of plasma volume [3—9] and in studies of albumin distribution [10] and vascular permeability [7, 8, 10]. Currently, spectrophotometric techniques for the determination of the concentrations of Evan's blue lack sensitivity and specificity. We report a method employing high-performance liquid chromatography (HPLC) for the rapid assay of Evan's blue in plasma samples. The method has distinct advantages over conventional techniques and will permit studies employing Evan's blue in a wide variety of clinical problems.

METHODS

Extraction of Evan's blue from plasma

A 150-μl sample of plasma collected in the presence of EDTA disodium salt, or a smaller volume of sample diluted to 150 μl with 154 m*M* sodium chloride solution, was placed in a microcentrifuge tube. The pH was increased to 11—12 by the addition of 120 μl of 0.1 *N* sodium hydroxide, after which 120 μl of magnesium oxide suspension (2 mg/ml) and 25 μl of Trypan blue solution (40 mg/l) were added. The mixture was agitated briefly, centrifuged in a high-speed table top centrifuge for 2 min, and the supernatant discarded. The pellet was washed twice with water by resuspending the pellet in 50 μl water, agitating, centrifuging, and discarding the supernatant. The pellet was then dissolved in 75 μl of 0.2 *N* hydrochloric acid, and the mixture neutralized with 75 μl of 0.2 *M* Tris. An aliquot of this solution was then injected in the HPLC system.

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High-performance liquid chromatography

The principal features of this system included an Altex 110A pump, a Rheodyne 7125 loop injector, an Alltech 300 × 5 mm, C_{18} reversed-phase column (10 μm particle size), Schoeffel SF770 variable-wavelength detector with tungsten lamp and a Beckman 10-mV strip chart recorder. The eluent was 66% (v/v) methanol and 34% aqueous phase (120 m*M* Tris, pH 8.0, 40 m*M* magnesium chloride) and was pumped at 1 ml/min. The effluent was monitored at 590 nm.

Quantitation of Evan's blue in plasma samples

Chromatographic standards of Evan's blue were made by appropriate dilutions of Evan's blue in 120 m*M* Tris, pH 8.0, 40 m*M* magnesium chloride, and standard curves were constructed by plotting peak height against concentration.

RESULTS

Evan's blue had a retention time of 5 min and produced a sharp symmetrical peak (Fig. 1). Trypan blue, added to the samples for visualization of the precipitate during the extraction procedure, was not retained on the column.

The relationship between chromatographic peak height and dye concentration was slightly non-linear for concentrations between 0.5 and 20 mg/ml: in a typical experiment, the ratio of peak height to concentration increased from 2.43 at 0.5 mg/l to 3.64 at 20 mg/l (Fig. 2). A linear plot of concentration vs. peak height visually appeared linear (r = 0.990) but was perceptably convex (Fig. 2A). The data were best represented by an exponential plot:

$$C = 10.0\ (P/P_{10})^{0.899}$$

where C is concentration (mg/l), P is the peak height for the sample, and P_{10} is the peak height of the standard containing 10 mg/l (Fig. 2B; r = 0.9996, n = 27). This formula was found to accurately describe peak height—concentration relationships on subsequent standard curve determinations. Four sets of concentration standards, each set containing 11—16 samples, had absolute sample mean errors (error/concentration) that ranged from 3.0 to 5.5%. Error was independent of concentration within the concentration range of 0.5—20 mg/l. The threshold of accurate detection (signal-to-noise ratio greater than 2) was 0.5 mg/l with a 0.050-ml injection volume (25 ng).

Recovery from extracted samples was 81.3% with a coefficient of variation of 4.8% (n = 16). Recovery was independent of concentration and recovery of samples from serum and 154 m*M* sodium chloride solution gave similar values.

No peaks with a retention time similar to Evan's blue have been observed in serum samples from subjects who had not received this compound. In addition, the following commonly used drugs were tested in the system and did not interfere with the determination of Evan's blue concentration: ampicillin, cephalothin, gentamicin, furosemide, acetazolamide, digoxin, caffeine, phenytoin, riboflavin, and indocyanine green. Twenty to thirty samples have been processed on a typical day.

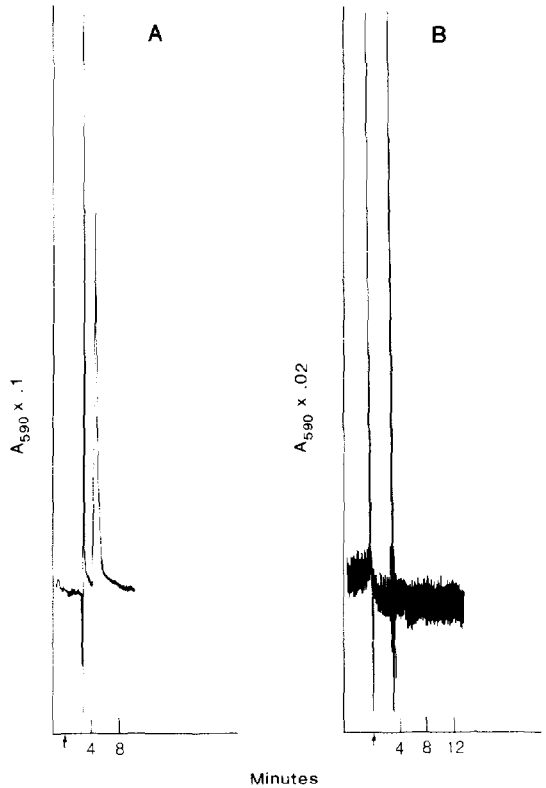

Fig. 1. HPLC of plasma samples. (A) Plasma containing 10 μg/ml of Evan's blue; attenuation 0.1. (B) Plasma containing no Evan's blue; attenuation 0.02. Plasma extraction procedures and chromatography conditions as described in text. In both cases injection volume was 0.050 ml. Injection performed as indicated at arrow; retention time of Evan's blue 5 min.

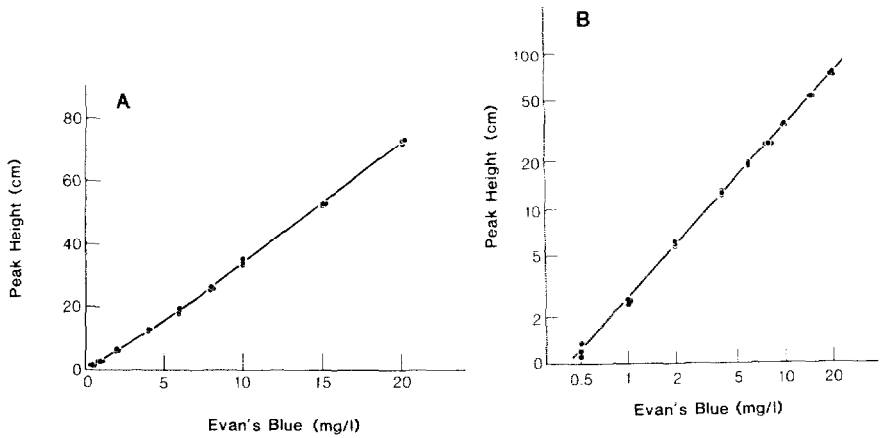

Fig. 2. Relationship between Evan's blue chromatographic peak height and concentration of dye injected (three points at each concentration, injection volume 0.050 ml; attenuation 0.04). (A) Linear plot: although linear plot fit of data is good (r = 0.990), there is a distinct convexity to the curve. Correspondingly, peak height to concentration ratios increase with concentration. (B) Logarithmic plot: fit is independent of concentration (r = 0.9996). Concentration accurately predicted by regression equation $C = 10\ (P/P_{10})^{0.899}$, where C is the concentration of the unknown, P is the peak height of the unknown and P_{10} is the peak height of a standard containing 10 μg/ml (see text).

DISCUSSION

Two analytical problems were encountered during the development of this assay technique. The first was the separation of Evan's blue from albumin in plasma, necessary because the Evan's blue—albumin complex had markedly different chromatographic properties from the free dye and since virtually all the dye in plasma (>99%) exists in the protein-bound form [2]. Conventional protein denaturation procedures did not quantitatively release free dye. This problem was solved by alkalinization of the plasma in the presence of excess metallic cation (Mg^{2+}) producing a dye lake that was insoluble in plasma. The principle of this procedure is similar to that used in methods in which other azo dyes are used for the quantitative determination of Mg^{2+} concentrations [11]. Once the lake was washed free of plasma proteins, the precipitate was redissolved in acid, the solution neutralized, and an aliquot chromatographed.

The second difficulty encountered was the non-linearity of the concentration—peak height relationship. Although the deviation from linearity was small, clinically unacceptable errors (greater than 5—10%) would be introduced at the lowest concentrations using linear assumptions. Alternatively, laborious, complete standard curves that do not assume any format, linear or exponential, could be developed with each day's determinations. We found, however, that the exponential formula experimentally developed and presented above gave excellent accuracy and precision (coefficient of variation of less than 5%) at concentrations achieved with clinically used doses of dye at 0.1—0.5 mg/kg. The likely explanation for the non-linearity is that increasing amounts of cation relative to dye decrease the affinity of the lake formation because of cation—cation interactions [12]. This hypothesis was supported by experiments in which we found a decreased recovery of dye from serum when the amount of magnesium oxide in the initial step of the extraction procedure was increased.

The clinical measurement of plasma volume is currently performed by determining the dilution volume of intravenously administered radiolabelled albumin, radiolabelled red cells, or Evan's blue. These techniques have been validated and protocols with normal values have been published [3—9]. The radioisotopic methods necessitate radiation exposure, which can be minimized by using larger blood sample volumes and avoiding repeated determinations in the same patient. The use of Evan's blue eliminates the radiation hazard and repeated determinations carry no hazard.

This technique presents significant advantages over other assay methods for Evan's blue. The high specificity of the method accrues from the extraction and chromatographic separation of the dye from other plasma constituents. Spectrophotometric methods are subject to interference in hemolyzed, turbid or lipemic sera [13]. Other attempts to eliminate the interference have involved the quantitation of the interfering substances [9] or more laborious chromatographic procedures [13]. The high sensitivity and accuracy of this assay procedure are due both to the lack of even minute amounts of interfering substances and to the high resolution characteristics of HPLC. This improvement in sensitivity over previous techniques will allow the reduction in sample size thereby permitting its application to even the smallest patient.

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