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MICROMETHOD FOR THE ANALYSIS OF EVANS BLUE IN PLASMA USING ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A micromethod for the determination of Evans Blue in plasma has been developed. Effective release of the dye from the dye—albumin complex was accomplished using a solution of urea and 2-mercaptoethanol. Ion-pairing techniques were used to extract the dye as well as the internal standard (Naphthol Blue Black B) from plasma. Separation involved ion-pair high-performance liquid chromatography. Eluent was monitored at 605 nm The technique results in a linear response over the concentration range of approximately 1.0—30.0 μg/ml. Assay performance was verified by estimating plasma volume in the rat.

INTRODUCTION

The estimation of plasma volume is of clinical importance. In addition, the results of basic research investigations can be greatly enhanced if size estimation of this space is possible. Evans Blue (T-1824) is an azo dye that avidly binds to plasma albumin [1], and hence is confined to the vascular space for a relatively long period following intravenous administration, Consequently, it has been successfully used as a marker compound for plasma/blood volume estimation [2–4].

The vast majority of plasma assay procedures hitherto developed for Evans Blue have depended upon spectrophotometric detection. Both single-wavelength and double-wavelength techniques have been reported [5, 6]. Generally, these procedures have involved some form of preparatory clean-up, e.g. acetone extraction [7], cellulose adsorption [8] or polyethylene glycol protein precipitation [3]. Recently, Green and Marchessault [9] reported the first and only assay procedure that employs high-performance liquid chromatography

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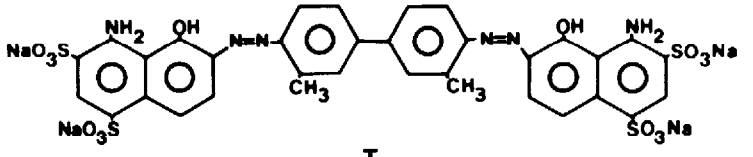
(HPLC). This technique, although an apparent improvement over previously reported methods, is characterized by a non-linear standard curve, a lack of internal standardization and considerable sample handling prior to analysis.

This report presents a new HPLC technique for the analysis of Evans Blue in plasma/serum. The method involves a release of the dye from albumin, ion-pair extraction of the dye from the biological matrix, followed by separation using ion-pair chromatography. Internal standardization has been incorporated into this procedure to reduce assay variability.

EXPERIMENTAL

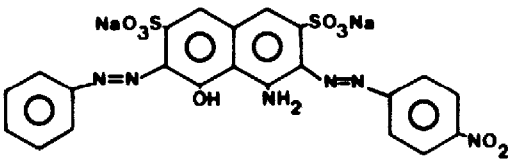
Analytical standards

Compound I (Fig. 1) was obtained from the United States Pharmacopeia Convention (Rockville, MD, U.S.A.). Both aqueous (1 mg/ml) and methanolic (2 μg/ml) solutions of this compound were prepared. Appropriate volumes of the aqueous solution were diluted with blank human plasma obtained from the Red Cross. The resultant concentrations ranged from approximately 1.0 to 30.0 μg/ml. Appropriate volumes of the methanolic solution were transferred to 100 mm × 13 mm extraction tubes fitted with PTFE-lined screw caps. These aliquots were evaporated to dryness using heat (35°C) and a gentle stream of nitrogen (N-EVAP, Organomation Assoc., South Berlin, MA, U.S.A.). The addition of 50 μl of blank rat serum/plasma to each of these samples was followed by vigorous mixing for 10 s (Vortex-Genie mixer, Scientific Industries, Bohemia, NY, U.S.A.). The concentrations of I in these samples were the same as those of the human standards.



I

EVANS BLUE



II

NAPHTHOL BLUE BLACK B

Fig. 1. Chemical structures of compound I (Evans Blue, T-1824, Direct Blue 53) and compound II (Naphthol Blue Black, Acid Black 1, Amidoblack 10B).

Compound II (Fig. 1) was purchased from Fluka (Hauppauge, NY, U.S.A.). An aqueous solution (15 μg/ml) was prepared and served as the working internal standard solution. This solution was stored at ambient temperature.

Reagents

The following reagents were used for the extraction and chromatographic separation of compounds I and II: urea, tetrabutylammonium dihydrogen phosphate (Aldrich, Milwaukee, WI, U.S.A.); 2-mercaptoethanol, tetrabutylammonium hydroxide (Sigma, St. Louis, MO, U.S.A.); acetonitrile (J.T. Baker, Phillipsburg, NJ, U.S.A.); chloroform (Waters Assoc., Milford, MA, U.S.A.); isopropanol (Burdick & Jackson Labs., Muskegon, IL, U.S.A.).

Extraction from plasma/serum

With the use of a microvolume glass syringe (Unimetrics, Anaheim, CA, U.S.A.), 50 μl of sample or standard were placed in a 100 mm × 13 mm extraction tube fitted with a PTFE-lined screw cap. A 50-μl volume of the working internal standard solution (II; 15 μl/ml in water) was added and the mixture vortexed. In a ventilation hood, 50 μl of a mixture of 2-mercaptoethanol—8 *M* urea (1:125) were added. Again the sample was vortexed. A 100-μl volume of 0.1 *M* aqueous solution of tetrabutylammonium hydroxide (pH 12.50) was added, followed by the addition of 3 ml of chloroform—isopropanol (83:17). The samples were tightly capped and shaken on a reciprocating shaker for 15 min. The tubes were then centrifuged at 500 *g* for 5 min. The upper aqueous layer was aspirated using a Pasteur pipet attached to a vacuum flask. The remaining organic layer was transferred to a clean disposable culture tube (75 mm × 12 mm) and subsequently evaporated to dryness using heat (35°C) and a gentle stream of nitrogen. Each evaporated sample was reconstituted with 200 μl of mobile phase.

Chromatography

The mobile phase consisted of 36% acetonitrile in phosphate buffer (0.067 *M*, pH 7.00). Sufficient tetrabutylammonium dihydrogen phosphate solution (1 *M*) was added to provide a final concentration of 0.005 *M*. Mobile phase was degassed by filtration. The flow through the column was maintained at 2.0 ml/min and the eluent monitored at 605 nm. Injection volumes varied from 40 to 100 μl depending upon the concentration in plasma. Peak heights for I and II were measured and calibration plots constructed, i.e. the ratio of the peak height of I and the peak height of II was plotted against the corresponding plasma concentration. The unknown concentrations of I of processed samples were estimated using linear regression analysis of calibration data.

Apparatus

The HPLC system consisted of a Model 510 solvent delivery pump and a Lambda Max Model 481 LC spectrophotometer (both from Waters Assoc.). Samples were introduced onto the column using a Waters Intelligent Sample Processor 710B (WISP). Times of peak absorbance were recorded on a linear chart recorder (Cole Parmer, Chicago, IL, U.S.A.) which had a paper speed of 0.25 cm/min. Full scale pen deflection corresponded to 0.02 absorbance units (a.u.f.s.).

A μBondapak C_{18} column served as the analytical column (30 cm × 3.9 mm I.D., 10 μm particle size, Waters Assoc.). A 5-cm guard column was manually packed with Bondapak C_{18}/Corasil (Waters Assoc.).

Reproducibility

Human plasma was spiked with compound I so that resultant concentrations fell in the range from 1.03 to 30.84 μg/ml. A set of spiked standards (including a blank sample) was extracted/chromatographed in the morning, at noon and in the late afternoon. This procedure was conducted on each of six days over a two-week interval. Standards were refrigerated between trials. The resultant peak-height ratios were analyzed for both between- and within-day effects on assay performance. In addition, these data were used to evaluate potential curvature in the plot of peak-height ratio versus plasma concentration.

Extraction efficiency

The efficiencies at which compound I and compound II were removed from plasma were evaluated at plasma concentrations of 5.14, 15.42 and 30.84 μg/ml for compound I. The concentration of compound II remained invariant, i.e. 50 μl of a 15 μg/ml aqueous solution were added to each 50 μl of spiked plasma. The peak height of each compound was compared to that of a corresponding sample made in mobile phase and injected directly. Corrections for dilutional effects of the assay procedure were made.

Stability

The stability of compound I in human plasma was evaluated at both refrigerated (1—5° C) and frozen temperatures (- 20° C). Standards (1.03—30.84 μg/ml) were placed in the respective storage areas, subsequently analyzed and the results compared to those obtained from freshly prepared standards. Statistical analyses for co-linearity were employed to detect degradation of compound I in stored samples.

In vivo trial

An aliquot (250 μl) of an aqueous solution of compound I (1.108 mg/ml) was intravenously injected via the external jugular vein into the right atrium of a female Sprague—Dawley rat. The weight normalized dose was 0.68 mg/kg. Blood samples (125 μl) were serially collected at 0 (prior to dose), 0.25, 0.50, 0.75, 1.0, 2.0, 3.0, 4.0 and 5.0 h after administration. The samples were allowed to clot and the serum harvested following centrifugation. All blood samples collected were replaced by an equal volume of normal saline. The serum samples were refrigerated overnight and analyzed for compound I content the next day. The plasma concentration versus time data were plotted semi-logarithmically. Plasma volume was estimated as the ratio of the administered dose and the back-extrapolated zero time concentration.

Statistics

All statistical analyses (analysis of covariance, bivariate regression analysis) were performed using a statistical analysis system (SAS Institute, Cary, NC, U.S.A.).

RESULTS

Representative chromatograms resulting from the analyses of blank and spiked human serum are presented in Fig. 2. No interfering endogenous substances were detected in either normal blank serum or serum harvested from hemolyzed (sonicated) whole blood. Baseline separation of compounds I and II is evident in the chromatogram for the spiked sample. The lower limit of sensitivity was 0.5 μg/ml at 0.02 a.u.f.s.

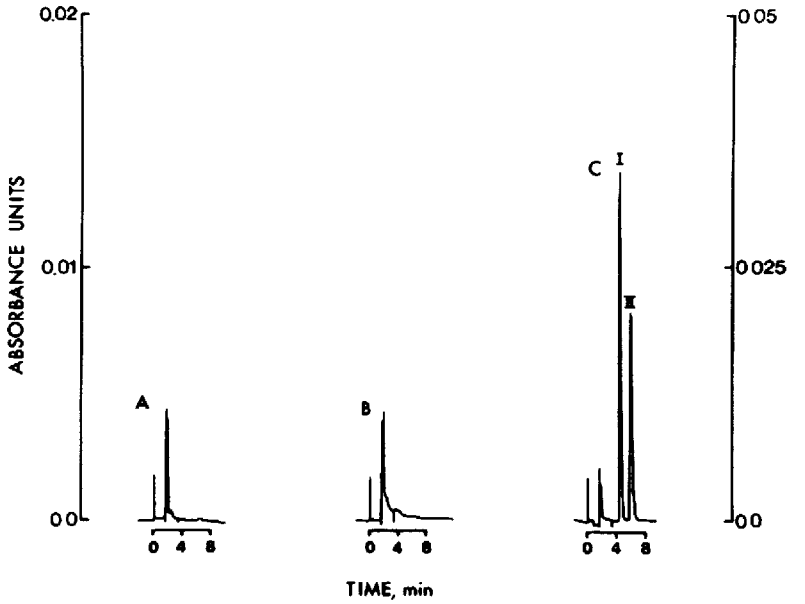


Fig. 2. Chromatograms of compounds I and II. (A) Blank human serum, 100-μl injection, 0.02 a.u.f.s. (B) Hemolyzed blank human serum, 100-μl injection, 0.02 a.u.f.s. (C) Spiked human serum (10.28 μg/ml), 100-μl injection, 0 05 a.u.f.s.

TABLE I

EXTRACTION OF COMPOUNDS I AND II FROM HUMAN PLASMA

Concentration (μg/ml)	*n*	Percentage of I extracted	C.V.* (%)	Percentage of II extracted	C.V. (%)
5.14	6	82.4	3.1	85.7	2.6
15.42	5	80.4	5.7	84.3	5.5
30.84	6	85.5	3.1	81.6	3.3

*C.V. = coefficient of variation.

The extraction efficiency for compound I from human plasma exceeded 80% over a six-fold range of concentrations (Table I). Compound II was extracted to a similar extent at each of the concentrations of compound I examined.

The relationship between the plasma concentration of compound I and the resulting peak-height ratio was linear (slope = 0.1796, intercept = —0.0402, r = 0.9998). The linearity of this relationship was supported by the statistical

TABLE II

ANALYSIS OF FACTORS AFFECTING REPRODUCIBILITY OF THE HPLC ASSAY OF COMPOUND I

c = concentration; d = days; t = time.

Source	Degrees of freedom	Sum of squares	Mean square	F
c	1	3.8176	3.8176	462.47*
c^2	1	0.0295	0.0295	3.57
c^3	1	0.0068	0.0068	0.82
Among days	20			
d	5	0.0011	0.0002	0.03
dc	5	0.0163	0.0033	0.39
dc^2	5	0.0042	0.0008	0.10
dc^3	5	0.0053	0.0011	0.13
Within day	8			
t	2	0.0018	0.0009	0.11
tc	2	0.0013	0.0007	0.08
tc^2	2	0.0100	0.0050	0.60
tc^3	2	0.0158	0.0079	0.96
Error	90	0.7429	0.0083	

*$P < 0.0001$.

analysis of the potential factors affecting assay reproducibility (Table II). As indicated in the table, inclusion of regression terms for curvature did not contribute significantly to the reduction of the error sums of squares. When curvature-related terms are excluded from the model, statistically significant differences were detected in the slopes of the standard curves from day to day ($p < 0.01$). The intercepts for the reduced model did not vary significantly from day to day. These results suggest that daily calibration curve preparation is necessary. As indicated by this analysis, the assay procedure was stable during any given day, i.e. no statistically significant differences were detected in standard curve slopes or intercepts within days. Within-day variability was low and relatively independent of concentration (coefficients of variation were 4.6% at 5.14 μg/ml and 3.1% at 30.84 μg/ml). The coefficients of variation for other standards were all less than 5%, except that for the 1.03 μg/ml standard which was 11.5%.

Human plasma samples spiked with compound I (1.03—30.84 μg/ml) were found to be stable at refrigerated temperatures (1—5°C) for at least 21 days. Following storage at −20°C for 7 days, no discernible degradation had occurred in plasma samples prepared to span the same concentration range presented above.

The performance of the assay was evaluated by estimating plasma concentrations of compound I in rat serum following an intravenous injection. Fig. 3 presents the chromatograms obtained from the serum samples collected just prior to dosing and 2 h after injection. The absence of interfering substances

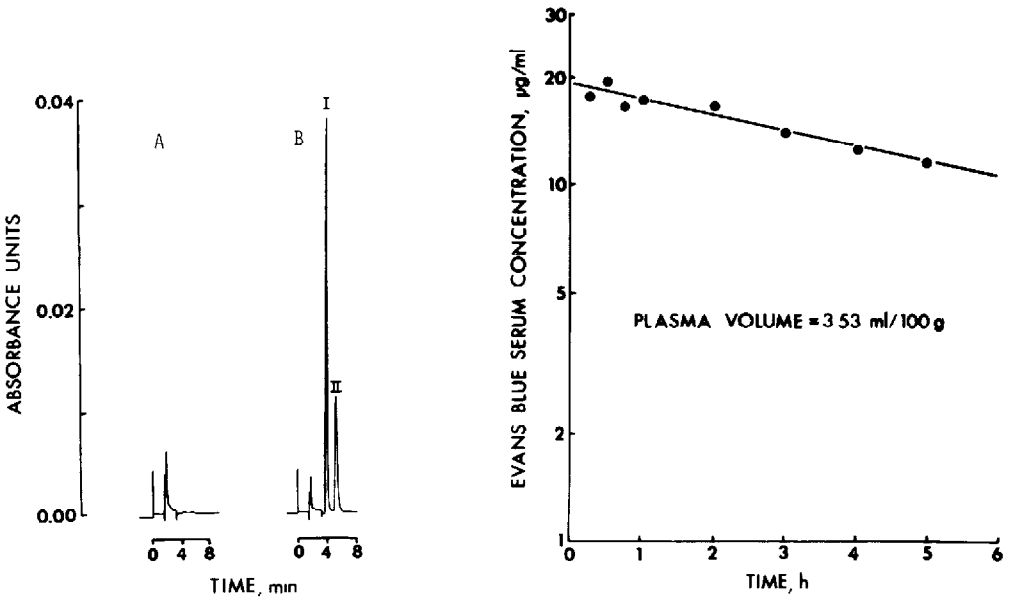


Fig. 3. Chromatograms of compounds I and II. (A) Blank rat serum collected prior to dosing, 100-µl injection, 0.05 a.u.f.s. (B) Rat serum collected 2 h after intravenous administration of I, 60-µl injection, 0.05 a.u.f.s.

Fig. 4. The logarithm of serum concentrations of compound I in the rat as a function of time following intravenous bolus injection. The solid line depicts the least-squares regression fit of all eight data points.

and excellent resolution of compounds I and II are evident. The relationship between serum concentrations of compound I in this animal and time is presented in Fig. 4. A log-linear decline in serum concentrations is evident. The plasma volume was estimated to be 3.53 ml per 100 g of total body weight.

DISCUSSION

The extraction of compound I from plasma was greatly hindered by the high affinity of the dye for albumin. The effective release of the substance from its binding sites on the albumin molecule proved to be very elusive. Commonly used denaturization techniques, e.g. sequential addition of saturated barium hydroxide/15% zinc sulfate solutions, heating, addition of acetonitrile, trichloroacetic acid solution, acetone and isopropanol were ineffective. These approaches resulted in extraction efficiencies from plasma of less than 30%. Green and Marchessault [9] reported similar difficulties. The addition of 15% benzalkonium chloride solution to plasma did result in substantial release of the dye. The use of this detergent has been successfully implemented in the spectrophotometric analyses developed by others [7]. However, co-extraction of the detergent from plasma resulted in its injection onto the HPLC system. Marked chromatographic perturbations ensued. Retention times for compound I progressively increased with each injection. This was accompanied by a general broadening of the chromatographic peak. Attempts to precipitate excess detergent with ammonium nitrate prior to extraction were successful.

However, the insoluble complexes redissolved in the organic extraction phase when it was introduced into the tube.

The presence of 2-mercaptoethanol was essential for adequate dye release. Presumably, this effect is mediated through the reduction of disulfide bonds in the albumin molecule. Attempts to release the dye using an aqueous solution of 8 *M* urea alone were unsuccessful. It should be noted that the extraction efficiency for compound I from phosphate buffer (0.067 *M*, pH 7.4) was of the same order as that from plasma. This suggests that the incomplete recovery from plasma is not likely due to incomplete release from the protein–dye complex, but rather to the limitations of the ion-pairing/extraction process.

Several attempts were made to extract the released dye into various organic phases without the aid of a counter-ion. Equal volumes of buffered solutions (pH 3.0—7.0) were added to plasma samples containing compound I in order to adjust pH prior to extraction. This approach did not result in appreciable transfer of dye to the organic phase. The presence of the tetrabutylammonium counter-ion was essential for substantial removal from plasma. No advantage was gained by placing excess ammonium sulfamate in the extraction tubes in an attempt to salt-out the ion pairs and hence enhance extractability.

The estimate of plasma volume (3.53 ml per 100 g) obtained using this procedure is in excellent agreement with those reported by others. Huang and Bondurant [4] used both radiolabeled albumin and compound I to obtain independent estimates of plasma volume in male albino rats (Wistar—Purdue). The values reported for the two approaches were 3.43 ± 0.48 and 3.36 ± 0.41 ml per 100 g of body weight, respectively. Others have reported similar values [10, 11].

The technique described herein offers many advantages over previously reported methods. The presence of hemoglobin in serum does not interfere with the detection of compound I. When grossly hemolyzed serum was analyzed, the areas in which compounds I and II eluted were totally free from interferences. This is in contrast to techniques that rely on direct spectrophotometric detection [3]. Clausen and Lifson [5] also reported that, when analyzing blood samples with volumes smaller than 500 μl, the optical density is a function of the amount of blood used. Variability in the measurements is attributable to the effects of blood colloids and salts on the absorption of light by the dye. In addition, spectrophotometric techniques generally require relatively large sample volumes [5—8]. Horowitz et al. [12] were able to use small volumes of rat plasma to quantify dye content colorimetrically, but specialized instrumentation was required.

An HPLC procedure is preferable to these techniques for a number of reasons. Firstly, it allows for the analysis of dye content in biological samples even when sample size is relatively small. Such a constraint routinely arises when small subjects/animals are involved and multiple samples are to be collected or when plasma volumes of small organs are to be estimated. Secondly, since a separation procedure is involved, variability in the analysis is reduced owing to the separation of endogenous substances prior to detection of the dye. Recently, Green and Marchessault [9] reported an HPLC technique. This procedure did not involve an extraction of the dye from plasma, but rather a

precipitation of the compound via the formation of a dye lake. Repeated washing/centrifugation of the precipitated pellet was necessary for adequate sample clean-up prior to injection. The extraction procedure presented in this report involves minimal sample handling and allows denatured protein to be quickly removed from the sample prior to injection. Generally, this approach provides for cleaner injected samples and consequently, longer column life. Green and Marchessault [9] also reported that the standard curve obtained over the concentration range of approximately 0.5—20.0 μg/ml was non-linear. This behavior was likely assay-specific and directly attributable to reduced dye lake formation at low plasma dye concentrations. A logarithmic transformation of the data was necessary in order to linearize the plot. No evidence of non-linearity was detected over the concentration range of 1.03—30.8 μg/ml for the procedure presented in this communication.

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