TECHNIQUES TO IMPROVE THE SPECIFICITY OF THE TRIHYDROXYINDOLE PROCEDURE[1]

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The trihydroxyindole procedure is a highly specific method for the determination of the adrenaline and noradrenaline content of biologic fluids such as urine and plasma, and it has therefore gained widespread popularity. However, the presence in urine of a number of as yet unidentified fluorescent contaminants of endogenous origin, as well as occasional exogenous contaminants (*e.g.*, aureomycin), can at times cause discrepancies between analyses performed fluorimetrically and those performed by bioassay techniques. These contaminants apparently are not eliminated completely by the usual aluminum hydroxide or aluminum oxide adsorption techniques, and they are not fully detected by the usual techniques of preparing fluorimetric blanks. In clinical practice, this problem of specificity of analyses threatens to become more acute as ever increasing varieties of new and potentially interfering drugs are made available.

Two general groups of techniques, which can be employed to increase the specificity of the trihydroxyindole procedure through the elimination of fluorescent contaminants, are the use of solvent extractions, and the more thorough use of aluminum hydroxide as an adsorbent. For instance, although adrenaline and noradrenaline cannot be readily extracted from aqueous solutions into organic solvents, the fluorescent trihydroxyindoles can be extracted with ease by a variety of such solvents. Therefore, increased specificity may be achieved by the extraction of fluorescent contaminants from urine prior to the typical aluminum hydroxide adsorption procedure, followed by the eventual isolation of the trihydroxyindoles by means of a second extraction with a fresh portion of organic solvent. Some of the extraction properties of adrenolutine are demonstrated in Table 1; similar results were obtained for noradrenolutine. It can be seen that the quantity of adrenolutine extracted into the organic phase (isobutanol) was dependent upon the pH of the aqueous phase: no fluorescent material was extracted at pH 12 whereas extraction was favored under acid conditions. Maximum intensity of fluorescence was observed in the isobutanol phase for extractions near pH 4.

The intensities and spectral distributions of trihydroxyindole fluorescence were affected by both the pH and the solvent. For instance, upon conversion of alkaline solutions of adrenolutine to neutral pH by addition of acid, a considerable decrease in intensity of yellow-green fluorescence was observed. On further acidification, an intense but blue-green fluorescence reappeared, with maximum intensity of fluorescence occurring in the range pH 4 to 5 (Table 1). In organic solvents such as isobutanol the trihydroxyindoles fluoresced a definite blue-green.

[1] The data presented herein were obtained in cooperation with Mr. Joseph Miranda of Seton Hall Medical College.

TABLE 1

*Extraction characteristics of adrenolutine**

	Galvanometer Deflections (Corrected for blanks)		
	Aqueous solution before extraction	Isobutanol extract†	Ethy lacetate extract†
pH 12	100	0	0
pH 6	61	35	—
pH 5	126	212	—
pH 4	122	220	125
pH 3	90	—	—
pH 2	43	—	—
pH 1	4	—	—

* Fluorescence was measured between 450 and 540 mμ with an Ilford 623 filter, and for an excitation of 405 mμ, on a Farrand fluorometer Model A.

† The aqueous phase was saturated with NaCl and extracted with an equal volume of organic solvent.

As a result of these spectral shifts, apparent changes in intensities of fluorescence were observed depending upon the particular choice of filters employed for measurements. For instance, as a result of the shift from yellow-green (pH 12) to blue-green, measurements obtained with the blue-green Ilford 623 filter indicated apparent increased intensities of fluorescence for aqueous solutions at pH 4 and for the corresponding isobutanol extract (Table 1), whereas with a yellow Corning 3486 filter apparent decreases in intensities of fluorescence were observed (data not shown).

The ability of organic solvents to extract the trihydroxyindoles increased with increasing polarity of the solvent: thus, ether was a weak extractant, ethyl acetate was better, and isobutanol was excellent. The comparative efficiencies of ethyl acetate and isobutanol are demonstrated in Table 1. For the extraction of an equal volume of sodium chloride saturated aqueous phase at pH 4, ethyl acetate possessed 50% the efficiency of isobutanol, while isobutanol was 100% efficient as judged by visual observations in ultraviolet light. The commercially available organic solvents as well as the sodium chloride were found to have deleterious effects on the stability of the trihydroxyindoles, as evidenced by fading fluorescence readings. However, stability could be reestablished by purification of the reagents, or by the addition of the antioxidant *l*-ascorbic acid (about 1 mg/10 ml of solution) to the aqueous trihydroxyindoles before extraction.

In organic solvents such as isobutanol, the fluorescence characteristics of adrenolutine differed sufficiently from those of noradrenolutine to permit simultaneous fluorimetric determinations to be performed in a manner analogous to that reported previously for aqueous alkaline solutions of the trihydroxyindoles.[2]

[2] Cohen, G. and Goldenberg, M.: The simultaneous fluorometric determination of adrenaline and noradrenaline in plasma. I. The fluorescence characteristics of adrenolutine and noradrenolutine and their simultaneous determination in mixtures. J. Neurochem. **2**: 58-70, 1957.

TABLE 2

*Excitation and fluorescence emission characteristics of adrenolutine and noradrenolutine in isobutanol**

Excitation Wave Length		Secondary Filter	Spectral Area Isolated	Galvanometer Deflections†			Ratio A/N
				A	N	Blank	
1	365 mμ	Corning 3486	530–800 mμ	100	55	15	2.1
2	405 mμ	Same	Same	100	28	4	4.0
3	436 mμ	Same	Same	100	16	4	8.0
4	365 mμ	Corning 3387 + 5443	455–520 mμ (peak at 470 mμ)	100	95	15	1.1
5	405 mμ	Same	Same	100	57	4	1.8

* 6.0 ml of aqueous solutions of the trihydroxyindoles (0.05 μg/ml) at pH 4.0 were saturated with NaCl and extracted with 4.0 ml isobutanol. Fluorescence measurements were performed on a Farrand fluorometer Model A.

† The fluorometer was arbitrarily standardized at 100 galvanometer deflection units (for each set of filter combinations) with the adrenolutine sample; then the fluorescence of the noradrenolutine sample and the blank were read.

For instance, in isobutanol (Table 2) the ratio of adrenolutine to noradrenolutine fluorescence increased with increasing excitation wavelength (*cf.* filter combinations 1, 2 and 3). Furthermore, lower ratios were noted for measurements obtained with an essentially blue filter combination such as Corning 3387 plus Corning 5443, than with a yellow filter such as the Corning 3486 (*cf.* combinations 1 and 4, or 2 and 5). Thus, simultaneous determinations of the trihydroxyindoles could be accomplished by choosing two sets of filters, each of which yielded a different fluorescence ratio for the trihydroxyindoles (*e.g.*, combinations 3 and 4), and by subsequently solving a set of simultaneous equations.

The adsorption of catechol compounds onto aluminum hydroxide or aluminum oxide has been the method of choice for the isolation of adrenaline and noradrenaline from plasma and urine. The specificity of this technique may be increased through further use of $Al(OH)_3$ at two stages in the procedure, namely after oxidation to adrenochrome and noradrenochrome, and again after formation of the trihydroxyindoles. Since the chromes are not adsorbed on $Al(OH)_3$, those fluorescent contaminants which are retained in the first adsorption but remain unchanged by oxidation (with MnO_2 or $K_3Fe(CN)_6$) may be conveniently adsorbed and removed at this stage. The trihydroxyindole tautomers of the chromes again contain the requisite catechol structure for adsorption onto $Al(OH)_3$ and therefore they can be separated by adsorption from those fluorescent contaminants which, after oxidation, are no longer capable of being adsorbed. The trihydroxyindoles may then be eluted by acid, or by phosphate ions (pH 6.5) which convert the $Al(OH)_3$ precipitate into insoluble $AlPO_4$, or by alkali (containing ascorbic acid) which converts the precipitate into soluble $Al(OH)_4^-$ (aluminate ion).

Phosphate ions may also be used for the elution of catecholamines (pH 4.0 to 6.5); elutions at pH 6.5 possess the advantage of avoiding the pH adjustment which is required to facilitate oxidation when elutions are performed with acid.

Another eluant which may be used with a great deal of ease and which, in addition, is highly specific for catechols, is $K_3Fe(CN)_6$. At pH 6.5, for instance, $K_3Fe(CN)_6$ will elute adrenaline and noradrenaline by converting them to their oxidized derivatives, adrenochrome and noradrenochrome. The particular advantage of this technique is that it accomplishes in a single step the same effect attained by subjecting separately isolated and subsequently oxidized extracts to a second adsorption, as previously outlined.

A tentative scheme for improvement of the specificity of the trihydroxyindole procedure, and based upon the techniques presented above, is as follows.

1. Fluorescent contaminants are extracted from urine (pH 4) into isobutanol.
2. Adrenaline and noradrenaline are adsorbed onto aluminum hydroxide.
3. Catecholamines are oxidized at pH 6.5 and thereby eluted, by the action of $K_3Fe(CN)_6$.
4. The trihydroxyindoles are formed by the addition of alkali (containing ascorbic acid) to the eluates of adrenochrome and noradrenochrome.
5. The trihydroxyindoles are extracted from aqueous solution (pH 4) into isobutanol.
6. The trihydroxyindoles are reextracted into aqueous solution (pH 7.5) which contains $Al(OH)_3$.
7. The $Al(OH)_3$ precipitate, which contains the adsorbed trihydroxyindoles, is dissolved in alkali (containing ascorbic acid).
8. Adrenolutine and noradrenolutine are then determined simultaneously and quantitatively by fluorimetry.

At this present writing, it has been found that although the scheme presented above is attractive from a theoretical standpoint, the cumulative losses occurring throughout the procedure result in recoveries of only about 50%. However, it is hoped that further study of the individual steps involved will, in the near future, improve the procedure to the point where the recoveries will be equally as respectable as the specificity achieved.