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Use of Ninhydrin to Purify Extracts of Putrefied Biological Specimens*

Milton Lessa Bastos**
Instituto Medico Legal, Rio de Janeiro, Brazil

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Summary. Highly impure residues obtained from an extract of alkalinized tissues can be purified by dissolving the residue in 5 ml of sulphuric acid (pH 5) and heating the solution on water bath with 0.1 g ninhydrin and 1.5 ml pyridine for one hour.

The reaction products are acidified (pH 1—2) with sulphuric acid and washed with ethyl acetate (2×30 ml). The washed aqueous phase is alkalinized and extracted with chloroform-butanol (9:1) (1×40 ml). The chloroform layer is washed with water (2×2 ml), filtered and evaporated to give a residue suitable for chromatographic analysis.

This purification procedure was applied to aliquots of an extract of putrefied tissues to which known amounts of atropine, brucine, caffeine, strychnine, heroin, hyoscine, morphine and quinine were added. Good recoveries were obtained (71—100%) when the added amount was over 1 mg. Fair recoveries were obtained with amounts in the range of 1 to 0.1 mg, except for atropine, hyoscine and quinine which gave low recoveries (15—30%).

For general application, the ninhydrin purification procedure must follow a previous ether extraction in basic medium to save the sympathicomimetic amines and some labile alkaloids.

Key-Words: Ninhydrin purification — Ninhydrinreinigung — Putrefied specimens — Fäulnisproben.

Zusammenfassung. Besonders unreine Rückstände von alkalischen Gewebsextrakten können dadurch gereinigt werden, daß der Rückstand in 5 ml Säure (pH 5) aufgenommen wird und die Lösung auf dem Wasserbad mit 0,1 g Ninhydrin und 1,5 ml Pyridin für 1 Std erhitzt wird. Das Reaktionsgemisch wird mit Schwefelsäure angesäuert (pH 1—2) und zweimal mit 30 ml Äthylacetat gewaschen. Die wäßrige Phase wird alkalisch gemacht und mit 40 ml Chloroform-Butanol-Gemisch (9:1) extrahiert. Die Chloroform-Phase wird zweimal mit 2 ml Wasser gewaschen, filtriert und für die chromatographische Analyse eingedampft.

Dieses Reinigungsverfahren wurde für Extrakte von fauligem Gewebsmaterial verwendet, dem bekannte Mengen an Atropin, Brucin, Coffein, Strychnin, Heroin, Hyoscin, Morphin und Chinin zugesetzt worden waren. Gute Ausbeuten (71—100%) wurden bei über 1 mg zugesetzter Substanzmenge, leidliche Ausbeuten bei 0,1—1 mg erzielt, mit Ausnahme von Atropin, Hyoscin und Chinin, die nur in geringer Ausbeute (15—30%) gewonnen werden konnten.

Für die allgemeine Anwendung empfiehlt es sich, das Ninhydrin-Reinigungsverfahren an eine vorherige Ätherextraktion aus alkalischem Medium anzuschließen, um sympathikomimetische Amine und einige labile Alkaloide zu schonen.

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** Laboratory for Addective Drugs, 520 First Avenue, New York, N. Y. 10016.

In the toxicological analysis of highly putrefied material a purification process designed to remove endogenous artifacts is essential if a sufficiently pure residue for the detection of exogenous substances is to be obtained. Usually the interfering substances may be eliminated by a purification process involving precipitation, differential and selective extractions and other troublesome operations. This invariably involves loss of time and material.

It is possible to neglect the stepwise purification process for the isolation of basic substances and extract all organic bases in a single operation. The "crude residue" obtained by evaporating the solvent used in such a procedure then may be purified. The choice of the solvent for this general extraction is very important. It affects both the recovery of alkaloids and purity of the "crude residue". Solvents such as isoamyl alcohol or chloroform-butanol extract many impurities which interfere with the chromatographic methods used to separate alkaloids. On the other hand, the use of a less general solvent may lead to an appreciable loss of some substances.

This paper describes a general method to purify the "crude residue" resulting from the extraction of basic substances from highly putrefied material following the use of chloroform butanol (9:1, v/v). The "crude residue" is dissolved in diluted sulphuric acid and then extracted at pH 10—12 with ether [1] and the ether is recovered. The aqueous phase is readjusted to pH 8—9 and then re-extracted with chloroform-butanol.

The residue obtained from the ether extraction is clean enough for chromatography and does not need further purification. The residue obtained from the chloroform-butanol extract contains many impurities whose presence make it difficult to detect all the substances that might be expected. A novel way to eliminate many of the endogenous basic substances from the residues of this chloroform-butanol extract was devised using ninhydrin. This procedure is described below.

Experimental Method

A. Preparation of Crude Residues

250 g of highly putrefied mixed viscera are acidified with acetic acid and heated [1]. Alternatively the aqueous suspension of viscera is saturated with ammonium sulphate following the procedure of Daubney and Nickols [2]. In each case the suspension is filtered and the filtrate is extracted twice with 150 ml of ether at pH 2. The aqueous phase is then alkalinized to pH 8—9 and extracted twice with 100 ml of ether at pH 2. This ether extract is not required for the analysis of organic bases and may be discarded. If the analyst is concerned with the detection of organic acids he may wish to save and use this extract for that purpose. The aqueous phase is then alkalinized to pH 8—9 and extracted twice with 100 ml of chloroform-butanol (9:1, v/v). This chloroform-butanol extract is evaporated to dryness. The resulting material is designated as the "crude residue".

B. Purification of the "Crude Residue"

The crude residue is dissolved in 5 ml of 0.1 N sulphuric acid, alkalinized to pH 12 with sodium hydroxide and extracted with 30 ml of ether. This ether is washed with water and the wash liquid is discarded. The ether extract contains some substances which would be destroyed by the subsequent ninhydrin purification procedure. The ether is evaporated and the residue

is dissolved in 1 drop of 0.1 N HCl. This solution is spotted on Whatman Number 1 paper and chromatographed, using butanol, acetic acid, water (4:1:5, v/v) as the developing solution.

The aqueous substrate from the ether extraction is adjusted to pH 5 and 100 mg of ninhydrin and 1.5 ml of pyridine are added. This mixture is heated on a water bath for one hour. After cooling to room temperature the dark solution is acidified with sulphuric acid to pH 1—2 and filtered into a 50 ml test tube. This solution is extracted twice with 30 ml of ethyl acetate. The supernatant ethyl acetate washes are discarded.

The purified aqueous phase is alkalinized to pH 8—9 with ammonia and then extracted with 40 ml chloroform-butanol (9:1, v/v). The aqueous supernatant solution is removed and discarded.

The chloroform-butanol extract is washed twice with 3 ml of water. The aqueous washes are discarded. The washed chloroform-butanol is filtered through a filter paper previously wetted with chloroform. The clear filtrate is then evaporated to about 3 ml. This is transferred to a 5 ml test tube and the evaporation to dryness is completed in a water bath.

The residue is kept overnight and then it is dissolved in one drop of 0.1 N HCl. This solution is then spotted on Whatman Number 1 paper and chromatographed using butanol, acetic acid and water (4:1:5, v/v) as the developing solution.

C. Preparation of a Pooled Crude Residue

A pooled crude residue was obtained from 10 kg of a mixture of putrefied viscera using the Daubney Nickols [2] method of extraction followed by the procedure described in A. The total residue was separated into several aliquots of 29 mg, each equivalent to 0.25 kg of viscera.

D. Alkaloid Recovery

The purification process described in B was evaluated by adding known quantities of alkaloids to an aliquot of the "crude residue" from the pool described above. This mixture was submitted to the purification process described in B and chromatographed. The alkaloid spot was localized by platinic iodide [3] or Ludy-Tenger reagent [4] and eluted from the paper by sodium sulphite-boric acid solution [5] or 2% EDTA solution in 1 N HCl [6].

The eluted solution was alkalinized and extracted with chloroform. The residue obtained by evaporation of this solvent was taken up in a pH 2 buffer and its ultraviolet absorption was determined. The reference solution was obtained by using material that was eluted from the adjacent portion of the chromatogram (Fig. 1) which contained only an aliquot of the "crude residue" that had been purified in the same way.

E. Choice of the Best Conditions for the Ninhydrin Clean up

The best conditions for the ninhydrin reaction was determined by comparison of the ninhydrin-pyridine reagent with other ninhydrin reagents. One ninhydrin reagent used 1 ml of glacial acetic acid and the other contained 10 ml of citrate buffer (1.765 g of trisodium citrate and 0.84 g of citric acid in 100 ml of water) instead of the pyridine. The process described in B was used. The U.V. absorption of the solutions thus obtained were compared between 220 and 320 mμ. This comparison was made by determining the area under each absorption curve and calculating the ratio of each obtained from the several clean-up procedures with that of the unprocessed "crude residues" (Rel. U.V.% in Table 1). The ability of these reagents to clean up the "crude residue" was measured using the methyl orange method for basic substances [7] and paper chromatography.

Results

The chromatogram of the "crude residue" may be seen in Fig. 1A. The absence of a large number of ninhydrin spots with lower R_f is due to the loss of the volatile amines during the evaporation of the solvent.

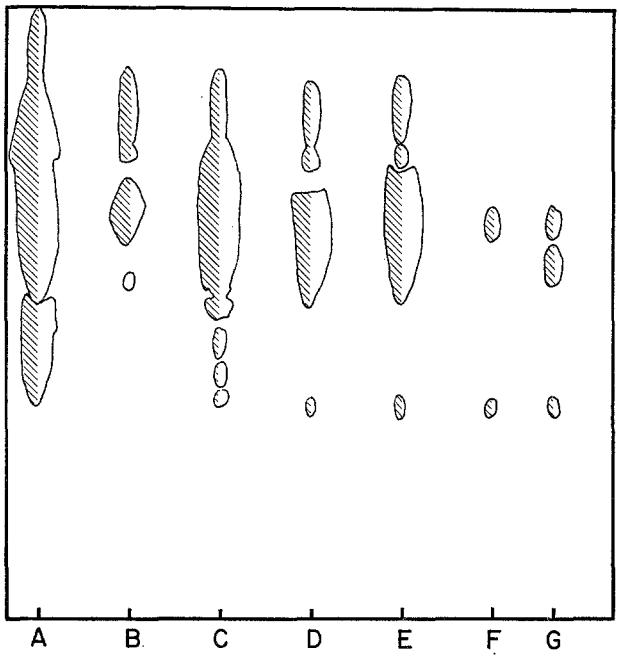

Fig. 1 A—G. Schematic representation of paper chromatograms obtained from an alkaline extraction of "crude residues" (solvent: butanol, acetic acid, water, 5:1:5, v/v; spray reagents: ninhydrin and iodoplatinate). The cross-hatched areas represent positive reaction with ninhydrin reagent. A Chromatogram of the "crude residue". B Chromatogram of the ether extract (pH 10—12) of the acid ether washed aqueous solution of the "crude residue". C Chromatogram of the purified chloroform-butanol extract obtained at pH 8—9, after employing the purification process described but without addition of ninhydrin. D The same as in C but employing ninhydrin and acetic acid. E The same as in C but employing ninhydrin and citrate buffer. F The same as in C but employing ninhydrin and pyridine. G The same as in F but after adding strychnine to the "crude residue" to check its recovery

Table 1

Clean-up reagent. 100 mg of ninhydrin with	Relative[a] U.V. (%)	Relative[a] absorption (methyl-orange 5%)	Chromato-graphy Fig. 1
2 ml pyridine	7.4	2.5	F
1 ml acetic acid	32.4	19.5	D
10 ml citrate buffer	26.8	31.6	E

[a] Relative to unclean "crude residue" as 100%.

The ninhydrin purification in the presence of pyridine gave the best result since it removed almost all the main impurities (Fig. 1 and Table 1).

The application of the ninhydrin-pyridine clean up to "crude residues" containing alkaloids gave good results. The chromatograms obtained were

reasonably free of endogenous interfering substances. This ninhydrin purification procedure permits easier identification of any alkaloids that may be present because the endogenous artifacts are minimized. It is worth while despite the loss of material during the purification process.

These losses were evaluated by determining the recovery of different amounts of alkaloids added to 29 mg of the "crude residue" and are reported in Table 2.

Table 2. *Recovery of drugs added to "crude residue" following ninhydrin clean up*

Drug	10.0—1.0 mg added	Per cent recovery	1.0—0.5 mg added	Per cent recovery	0.5—0.1 mg added	Per cent recovery
Strychnine	8.4	100	0.7	82		
Brucine					0.5	60
Quinine	1.1	71			0.03	16
Codeine	1.2	72	0.5	89		
Morphine			0.7	53	0.3	49
Caffeine			0.5	63		
Atropine	7.2	90			0.2	12.3
Heroin	1.2	76				
Hyoscine			0.5	31		

A good recovery was obtained (71—100%) when more than 1 mg of added drug was used; fair recovery (49—89%) in the range of 0.1 mg to 1 mg except for atropine and hyoscine. This fact may be explained by the weakness of the esther linkage of these alkaloids.

Discussion

Ninhydrin is known to react with primary and secondary amines which are bound to alipathic radicals, with piperidin-carboxilics [5] and with some guanidinium compounds [8].

The main reaction occurs with primary amines. Secondary amines, such as ephedrine, adrenaline and sarcosine react after a preliminary degradation mechanism which then produces primary amines [9].

Ninhydrin reacts with primary amines in two ways [10]:

a) by oxidation of the amines to aldehydes to form hydrindantine. The aldehyde thus formed may react with a diamine to form a purple product — Ruhemann purpur formation [11]. Other condensation products may be produced as in the case of tryptamines in acid medium. The aldehydes produced also may react with products such as serotonin, producing yellow-green fluorescent products [12];

b) by Schiff base formation, through the condensation in the pyridine medium of the ninhydrin carbonyl group with the amine group [13].

The mechanism of the clean up of impure residues may be explained by the reaction (b) above. The Ruhemann purpur formation peculiar to reaction (a) was observed in the first phase of the purification process and was probably due to the diamines present in the residues.

The condensation reaction of the aldehydes formed with some active methylene group, to produce fluorescent compounds, depends on the acid concentration [12] since it does not occur in pyridine medium.

There are some anomalous reactions of ninhydrin with carbonyl groups [14] but ninhydrin may be considered inactive against those substances that may be of interest to the toxicologist with the exception of ephedrine, desoxyephedrine, amphetamine, harmine and a few other similar compounds.

The ninhydrin clean up is not a general procedure as its application is limited to impure residues after a previous separation of the sympathicomimetic amines and other aliphatic amine compounds by ether extraction, distillation or diffusion technics.

The aldehyde formed during the reaction will not react with drugs having active hydrogen sites. Had this occurred, the recovery of codeine and morphine would have been very low.

As a result of the ninhydrin clean-up process, and according to our experience, any general solvent such as chloroform-butanol (9:1) may be used to extract the basic substances from putrefied material. The crude residue obtained may be dissolved and extracted with ether (pH 10—12) to save the sympathicomimetic amines and atropine, hyoscine, quinine and other ester alkaloids [1]. This ethereal extract is sufficiently pure for further chromatographic separation (Fig. 1, Chromatogram B).

The aqueous phase containing the basic and amphoteric substances in amounts over 0.1 mg may be treated by the above clean-up procedure with fair recovery. The chromatograms obtained from the purified residues are free from substances that will be developed by the iodoplatinate reagent.

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Professor M. L. Bastos
Instituto Medico Legal
Rio de Janeiro, Brazil