Biochemical Pharmacology, 1963, Vol. 12, pp. 445–450. Pergamon Press Ltd., Printed in Great Britain.

A METHOD FOR THE DETERMINATION OF MICROQUANTITIES OF IPRONIAZID AND OTHER N_1-ALKYL-N_2-ACYL-HYDRAZINES IN BODY FLUIDS AND TISSUES

J. RIEDER and M. ROTH

Department of experimental medicine of F. Hoffmann-La Roche & Co. Ltd., Basle.

(*Received* 12 *December* 1962; *accepted* 4 *February* 1963)

Abstract—A method for quantitative determination of iproniazid, isocarboxazid and pivaloyl-benzylhydrazine, as well as some other compounds of the N_1-alkyl-N_2-acyl-hydrazine type, in all kinds of biological material is described. It is based on the extraction of the compounds into a butyl acetate-butanol mixture or (for urine only) chloroform, and subsequent formation of a coloured formazan by reaction with a tetrazolium salt-reagent. The procedure is relatively simple, sensitive, well reproducible and sufficiently specific for pharmacokinetic studies of the drugs mentioned.

OUR studies on the fate of iproniazid*, isocarboxazid† and pivaloyl-benzyl-hydrazine‡ —drugs which are used in psychiatry and cardiology—in the animal organism have necessitated the elaboration of methods for their estimation in biological materials. In the present paper a procedure suitable for all these compounds and also some other N_1-alkyl-N_2-acyl-hydrazines is described.

Other such methods have been published only for the determination of iproniazid. (Rubin *et al.*[1]), converted the drug to isonicotinic acid and estimated this substance colorimetrically by a reaction with cyanogen bromide and ammonia; Jacobs[2] used the reduction of potassium ferricyanide and subsequent formation of Turnbull's blue; Hess and Weissbach[3] measured the u.v. absorption and the colour produced with a phosphotungstic and phosphomolybdic reagent.

PRINCIPLE

Samples of body fluids or extracts of tissue homogenates in a buffer of pH 3·0 are saturated with sodium sulfate and magnesium oxide and then extracted by shaking with butylacetate–butanol (or chloroform for urine only). An aliquot of the organic extract is treated with a solution of blue tetrazolium and KOH in methanol under standardized heating conditions. The tetrazolium salt is reduced by the hydrazine-moiety of the drug with formation of the purple coloured formazan, the concentration of which is determined by spectrophotometry.

(The applicability of the tetrazolium salt—which is a long-known reagent for many reducing compounds—for the estimation of iproniazid was first demonstrated by

* = 1-Isonicotinyl-2-isopropylhydrazine = Marsilid ®.
† = 1-Benzyl-2(5-methyl-3-isoxazolylcarbonyl)-hydrazine = Marplan ®.
‡ = 1-Pivaloyl-2-benzylhydrazine = Tersavid ®.

L. O. Randall and V. Iliev, Roche, Nutley, 1957, using an otherwise wholly different analytical procedure; unpublished communication).

REAGENTS

All substances mentioned should be of analytical grade.

McIlvaine Buffer of pH 3·0

100 ml of this are prepared by mixing 79·45 ml 0·1 M solution of citric acid in aqua dest. with 20·55 ml 0·2 M solution of disodium phosphate in aqua dest. The mixture, although stable at room temperature for a long time, should nevertheless be stored at +2° C to avoid mould formation.

Butyl Acetate–Butanol Solvent

A mixture of 9 volumes of butyl acetate pro anal. + 1 volume of n-butanol pro anal. is best. However a commercially available quality of butyl acetate, contaminated with varying amounts (10–15%) of butanol but otherwise pure, proved to be quite satisfactory.

Mixture of Sodium Sulfate and Magnesium Oxide

It is prepared by mixing carefully up to complete homogenization 30 parts by weight of finely powdered Na_2SO_4 siccum and 1 part by weight of finely powdered MgO.

Tetrazolium Reagent

It consists of a 0·125 per cent solution of 3:3′-dianisole-bis-4:4′-(3:5-diphenyl)-tetrazolium chloride in methanol. The liquid should be stored at +2° C and exposed to light as little as possible. Under such conditions it can serve for at least one month. We use a pure quality of the tetrazolium salt obtained from the British Drug House Ltd., in Poole (Great Britain).

Alkalizing Reagent

It consists of a 0·02 N solution of potassium hydroxide in methanol. When kept at +2 °C the solution is stable for at least one month.

Chloroform

PREPARATION AND CONSERVATION OF BIOLOGICAL SAMPLES

Blood and other coagulable body fluids are rendered uncoagulable by the addition of potassium oxalate (1·5 mg/ml). The blood plasma should be separated without delay. If this is impracticable, the blood must be kept at +2 °C until centrifuged.

Non-coagulable fluids do not need any preparation except, possibly, elimination of suspended solids by centrifugation.

Organs must be excised immediately after death by exsanguination, and should be frozen at once, in order to avoid enzymatic alteration of the drug. They are thawed again only directly before analysis.

Freezing at −18 °C is also suitable for the conservation of liquid material prior to analysis. Deep-frozen samples showed no measurable diminution of their iproniazid content within 3 weeks.

PROCEDURES

Determination of Iproniazid, Isocarboxazol and Pivaloyl-Benzylhydrazid in Body Fluids

All kinds of liquid biological samples can be treated alike (some exceptions for urine are indicated under Nos. 2, 3, 8a and 9 below):

1. 0·5 ml of the fluid sample is pipetted into a 15 ml shaking tube with glass stopper.
2. 1·5 ml pH 3-McIlvaine buffer is added. For urine distilled water is used instead of the buffer. After mixing, the tube is allowed to stand for 10 min.*
3. 4·0 ml of butyl acetate–butanol mixture is added, *without stirring*. For urine only, this solvent is replaced by 5 ml chloroform.
4. Addition of 0·75 gm of the Na_2SO_4/MgO-mixture in order to shift the pH to 10 and exert a salting-out effect. The tubes are immediately shaken repeatedly by hand, to prevent the sulfate from caking.
5. The tubes are shaken for 30 min by machine.
6. They are centrifuged until the phases separate (about 20 min at 2400 turns/min).
7. 3 ml of the supernatant phase are transferred to a test tube. 0·1 ml tetrazolium reagent, and 0·1 ml alkalizing reagent are added and the tube shaken for mixing. It should not be exposed to direct sunlight.

8a. All test tubes of one series (except those containing urine extracts) are placed in a support and immersed for 1 min in boiling water. At the end of the heating time the support is immediately plunged into cold water for cooling down to room temperature. The purpose of the heating operation is to accelerate the formation of the coloured formazan and to bring its extinction to a constant level.

8b. The chloroform extracts obtained from urine samples are not treated as described under 8a, but are placed for 20 min in a water bath at 20 °C.

9. The extinction of the solutions is measured in 1 cm layer at 525 mμ, using any spectrophotometer. This has to be done for urine extracts as soon as the 20 min mentioned under 8b have elapsed, since the formazan-colour is not stable in chloroform; for all other extracts within 30 min after cooling according to 8a.
10. The sample-blank and the reagent-blank are determined separately (see specifications under "Calculation of Results").

Estimation of the Drugs in Tissues

We prefer to work with whole organs, rather than parts, in order to avoid errors due to inhomogeneous distribution of the substances to be studied.

1. The tissues are minced if necessary with bent scissors and then homogenized in an ice-cooled Potter-type glass-homogenizer. Per g of the larger organs (as liver, kidney, brain, heart) 5 ml pH 3·0-McIlvaine buffer are added. For small organs (as spleen or adrenals) of small animals we use 5–15 ml of the buffer per g, in order to gain after centrifugation a volume of aqueous supernatant sufficient for analyses in duplicate. The buffer will stop the activity of tissue-enzymes, which otherwise could possibly alter the drugs.
2. The homogenized tissue-buffer-suspensions are transferred to 15 ml shaking tubes and shaken by machine for 30 min. They are then centrifuged at 2400 rotations per min, for liver samples 30 min, for samples of other organs 15 min.

* This step is advantageous in spite of the succeeding alkalinization, since for protein-containing samples it provides a higher recovery as well as a denser packing of the protein sediment and therefore facilitates subsequent isolation of the organic extract.

3. 2 ml of the supernatant are pipetted off and transferred to a new 15 ml shaking tube.
4. The procedure then continues as for liquid samples described above. (Steps 3 to 10).

In cases where extinction values of the final solution fall beyond the straight part of the calibration curve when this normal procedure is used, the sample is diluted appropriately before a new analysis.

CONSTRUCTION OF A CALIBRATION CURVE

For the preparation of the calibration solutions, the butylacetate–butanol or chloroform must be pretreated, by shaking 2 volumes of each with 1 volume of pH 3-McIlvaine buffer plus 0·375 g of Na_2SO_4/MgO powder mixture per ml of solvent for 30 min, as is done with the samples.

Three millilitres of each calibration solution are analysed by the procedure described above for biological fluids.

Under our working conditions the calibration curves are found to be straight up to concentrations of 6 μg/ml of calibration solution, and then begin to fall (see example in Fig. 1).

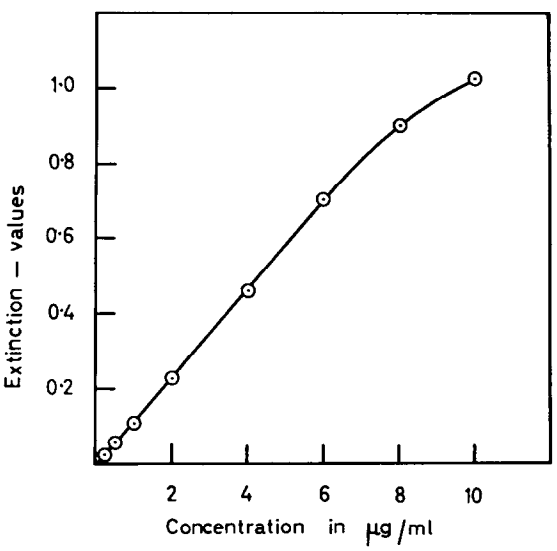

FIG. 1. Calibration curve for the determination of Marsilid-base.
Solvent: Butyl acetate–butanol mixture (87:13), pretreated by shaking with ½ volume of pH 3-McIlvaine buffer + Na_2SO_4 sicc + MgO in surplus.
Photometry: at wavelength 525 mμ in 1 cm layer.
Extinction coefficient in the range between 0 and 6 μg/ml = 9·0.

CALCULATION OF RESULTS

The concentrations of the hydrazides in the samples are calculated as usual on the basis of the total extinction of the measured solutions minus sample-blank minus reagent-blank.

Sample-blanks, obtained from untreated controls, were found in guinea pigs to have very low extinction values, varying for blood plasma, liver and brain between 0 and 0·010.

The reagent-blank must be determined daily owing to the slow alteration of some reagents (especially the tetrazolium salt solution when left at room temperature and exposed to light). Under our working conditions the reagent-blank, obtained by using distilled water in place of a sample throughout the analytical procedure and reading against the pure solvent of the final solution, was found to vary between 0·035 and 0·050 extinction units.

SOME FEATURES OF THE METHOD

Sensitivity

The minimal concentration of iproniazid, isocarboxazid or pivaloyl-benzyl-hydrazid that can be measured with the method described is 1 μg/ml or g of biological material. The minimal sample size needed is 0·5 ml or g, if measuring occurs in cuvettes of 10 mm internal length. The named sensitivity is adequate for experiments with laboratory animals receiving single intraperitoneal doses of at least 10 mg iproniazid, 50 mg isocarboxazid or 20 mg pivaloyl-benzylhydrazid per kg.

For blood level investigations in humans treated with usual therapeutic doses of iproniazid (1–2 mg/kg) and pivaloyl-benzylhydrazid (1·5–4 mg/kg), the sensitivity of the method is needed up to its limit. For studies on the fate of isocarboxazid in man our procedure would scarcely be sensitive enough, since this substance is normally given in doses of only 0·5–1·0 mg/kg daily and is readily metabolized.

Recoveries

The recoveries obtained after addition of known quantities of the drugs to biological samples are of the following orders (concentrations indicated in brackets):

TABLE 1.

Preparation	Urine	Blood plasma	Liver	Brain
Iproniazid	90% (10–40 μg/ml)	93% (2·5–10 μg/ml)	90% (2·5–10 μg/g)	93% (2·5–10 μg/g)
Isocarboxazid	94% (10 μg/ml)	93% (20 μg/ml)	70% (20–40 μg/g)	— —
Pivaloyl-benzylhydrazine	92% (200 μg/ml)	99% (20 μg/ml)	70% (20 μg/g)	96% (10 μg/g)

Reproducibility

This was examined for iproniazid by making 10 analyses of the same samples of blood plasma and liver respectively. The means and standard deviations of concentrations were found to be:

for plasma: mean = 38·06 μg/ml; s = 0·23 μg/ml
for liver: mean = 32·53 μg/g; s = 0·40 μg/g.

Thus the reproducibility can be considered to be excellent.

Specificity

In physiological concentrations none of the normal constituents of the mammalian organism will disturb the estimation of drug concentration by the method described. This is clearly shown by the very low sample-blanks quoted under "Calculation of Results".

Our present experience indicated that the procedure will enable the determination not only of iproniazid, isocarboxazid and pivaloyl-benzylhydrazid, but also of some other N_1-alkyl-N_2-acyl-hydrazines.

Concerning concomitant reactions with metabolites of the drugs, the following statement can be made:

Among all known metabolites of iproniazid[4] only isonicotinic acid hydrazide (=isoniazid) is measured simultaneously by our method. However, isoniazid appears only in small amounts in urine after iproniazid medication and gives extinctions only 1/7 as high as equimolar concentrations of iproniazid. Acetyl-isoniazid, isonicotinic acid, and alkylhydrazines, such as isopropylhydrazine or benzyl-hydrazine, do not yield coloured products with our techniques. Hydrazine does react with blue tetrazolium, but is not extracted by the organic solvents.

In conclusion, the method presented here is sufficiently specific for the unchanged drugs. The specificity is of course not due to the colour formation with the tetrazolium salt as such (since a similar reaction occurs with many other reducing substances) but to the conditions under which it takes place and the foregoing extraction procedure.

RESULTS

As an example of results obtained by the described method we add the following table:

TABLE 2. CONCENTRATIONS OF IPRONIAZID, ISOCARBOXAZID AND PIVALOYL-BENZYL-HYDRAZID IN BLOOD PLASMA, LIVER, HEART AND BRAIN OF RATS (WEIGHING 80–100 g) AFTER A SINGLE I.P. INJECTION OF 417 μMOLE/KG (— EQUIMOLAR TO 75 MG/KG IPRONIAZID BASE) OF THE DRUGS. FOR EACH ITEM THE SAMPLES FROM 4 RATS HAVE BEEN POOLED.

The values are not corrected for uncomplete recovery depending on the sample type (see Table 1).

Drugs	Hours after medication	Blood plasma mμmole/ml	Liver mμmole/g	Heart mμmole/g	Brain mμmole/g
Iproniazid (M.W. = 180)	1	332·1	266·5	290·7	305·5
	2	176·6	163·3	192·2	159·5
	3	182·6	153·4	141·1	118·4
	4	172·8	159·5	155·5	126·1
Isocarboxacid (M.W. = 231·2)	1	40·8	67·4	38·9	32·7
	2	19·4	44·5	27·0	17·1
	3	6·9	24·9	15·6	11·9
	4	6·1	6·7	18·9	8·3
Pivaloyl-benzyl-hydrazin (M.W. = 206·28)	1	236·5	365·0	122·2	142·6
	2	91·1	240·0	51·4	52·8
	3	8·1	43·6	34·9	22·7
	4	7·4	19·8	9·0	30·8

REFERENCES

1. S. H. RUBIN, L. DREKTER, J. SCHEINER and E. DERITTER, *Dis. Chest*, **21**, 439 (1952).
2. M. B. JACOBS, *Science*, **118**, 142 (1953).
3. S. HESS, H. WEISSBACH, B. G. REDFIELD and S. UDENFRIEND, *J. Pharmacol. exp. Ther.*, **124**, 189 (1958).
4. B. A. KOECHLIN and V. ILIEV, *Ann. N.Y. Acad. Sci.*, **80**, 864 (1958).