Fluorometric Assay of Bethanidine in Plasma

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Abstract □ A fluorometric method for determining bethanidine in blood plasma is described. The bethanidine is extracted into chloroform, a drug–dye complex with eosin Y is formed, and the fluorescence is measured (excitation, 535 nm; fluorescence, 560 nm). The assay detects 0.02 μM bethanidine (4 ng/ml) in plasma. The relative fluorescence of several body constituents and antihypertensive drugs is negligible. The plasma levels of bethanidine in four hypertensive patients receiving this drug were measured.

Keyphrases □ Bethanidine—fluorometric assay in plasma, complexed with eosin Y □ Fluorometry—analysis, bethanidine in plasma

Bethanidine is a new antihypertensive drug of the guanidine class (1). It is an adrenergic depleting agent similar to guanethidine, but the effect is of a shorter duration. Most potent antihypertensive compounds, including bethanidine, exhibit considerable variation in the dose necessary for acceptable control of blood pressure in different individuals. A simple fluorometric assay for bethanidine in plasma was developed in the present study. Bethanidine is extracted from plasma into chloroform, and the fluorescence then is determined using a drug–dye complex formed with eosin Y. This simple fluorometric assay will make it possible to study blood levels and pharmacokinetic parameters of bethanidine in hypertensive patients and to correlate blood levels with therapeutic efficacy.

EXPERIMENTAL

Analytical grade chloroform[1] stabilized with 0.75% ethanol was used without purification. The commercial source was important. Eosin Y[2] was stored in 0.1 M sodium phosphate buffer, pH 7.4, and was stable indefinitely. Bethanidine[3] was chromatographically and spectrophotometrically pure. Radioactive ^{14}C-bethanidine[4] (2.37 mCi/mmole) labeled at the benzyl carbon was purified by TLC on precoated silica gel plates[5]. In an ascending solvent system of butanol–acetic acid–water (3:1:1), the developing time was 6 hr at 25°. Unlabeled and ^{14}C-bethanidine had an R_f value of 0.53. There was a radioactive contaminant, R_f 0.57, in the ^{14}C-bethanidine. The 0.53 band was extracted into chloroform which then only contained ^{14}C-bethanidine. Fenfluramine[3], guanethidine[6], spironolactone[7] and its diethioacetylated-Δ^6 derivative[8], hydrochlorothiazide[9], and benzthiazide[3] were analytically pure. All other chemicals, reagents, and materials were of reagent grade[10].

Radioactivity was determined by liquid scintillation[11]. Plasma or urine (0.2–0.5 ml) was mixed with 1.5 ml of solubilizer. Then 0.5 ml of 30% H_2O_2 was introduced and allowed to incubate 30 min at 23°; finally, 15 ml of scintillation mix was added. Quenching was corrected by introducing ^{14}C-bethanidine as an internal standard after the initial counting.

Four hypertensive patients (age 45–52, weight 60–76 kg, three female and one male) were maintained on 50 mg of benzthiazide twice daily and 20 mEq of potassium chloride for 1 week before and during the bethanidine study. The patients gave informed written consent. At the first dose of bethanidine, they had a supine systolic blood pressure of 183 ± 16 mm Hg and a diastolic pressure of 119 ± 12 mm Hg. The patients are representative of a larger group under investigations for blood levels and pharmacokinetic parameters[12]. From a therapeutic standpoint, the bethanidine was given three times daily at varying doses and it was necessary to reduce the benzthiazide to 50 mg daily after 1 month.

Venous blood was collected in chilled 7-ml draw vacutainer tubes containing 10.5 mg ethylenediaminetetraacetic acid for plasma or into vacutainer clot tubes for serum and stored on ice. Preliminary studies utilized heparin tubes, which gave slightly higher blanks (2). Within 1 hr, the samples were centrifuged at 2000×g for 10 min and the supernates were transferred to specially cleaned test tubes and stored at −90°. The temperature and time were not critical for the bethanidine assay, but these conditions preserved the samples for analyses of other labile constituents.

Steps I–III of the assay were carried out in 1.6 × 14-cm, specially cleaned, glass-stoppered tubes protected from light with aluminum foil. The tubes and glassware were cleaned by boiling for 15 min in 0.1 N NaOH, rinsing, boiling for 15 min in 10% HCl, rinsing in water, boiling in distilled water for 15 min, rinsing three times in double-distilled water, and finally drying at room temperature. Furthermore, all steps (I–IV) were done in a cool (23–25°) dimly lighted room; the sample analyses were uniformly timed, within and between steps, to minimize adverse effects of chloroform degradation and of air and light exposure on net fluorescence and sensitivity. Reagents were prepared with double-distilled water.

Step I—The sample (2 ml) and 0.4 ml of 0.5 M sodium borate buffer, pH 10.5, are mixed and then 10 ml of chloroform is introduced. The tubes are tightly stoppered and wrapped in aluminum foil. Extraction is carried out for 10 min on a mechanical circular agitator. The stoppered samples are centrifuged at 500×g at 23° for 10 min; the upper aqueous portion contains an opalescent upper layer and a particulate layer at the chloroform interface.

Step II—The opalescent layer (1.5 ml) is transferred to a second tube containing 0.2 ml of 5 N NaOH, and then 7 ml of chloroform is added. The mixture is agitated and centrifuged as before.

Step III—The aqueous layer is aspirated and discarded, and 5 ml of the chloroform layer is transferred to a third tube containing 1 ml of 4×10^{-4} M eosin Y in 0.1 M sodium phosphate buffer, pH 7.4. The mixture is agitated and centrifuged as before.

Step IV—Fluorescence is measured[13] in 10 × 10 × 44-mm cells in a darkened room. Approximately 0.5 ml of the upper aqueous eosin layer is aspirated into a 5-ml pipet, and the tip is lowered to aspirate 3 ml of the chloroform layer containing the bethanidine–eosin complex. The contents of the pipet are transferred to the cell in such a way that the chloroform layer enters followed by the eosin layer; the chloroform layer must be completely covered by the eosin layer. The light path of the cell is visually inspected for small droplets of eosin, which can interfere with the recording. The fluorescence is determined at 560 nm with excitation at 535 nm. Readings must be made immediately after the sample is placed into the fluorometer due to rapid decay of fluorescence in the exci-

[1] Mallinckrodt Chemical Co., St. Louis, Mo.
[2] Aldrich Chemical Co., Milwaukee, Wis.
[3] A. H. Robins Co., Richmond, Va.
[4] New England Nuclear Corp., Boston, MA 02118
[5] E. Merck A. B., Darmstadt, Germany.
[6] Lot L-5746, [2-(hexahydro-1-(2H)-azocinyl)ethyl]guanidine sulfate, Ciba Pharmaceutical Co., Summit, NJ 07901
[7] Lot SC-9420, G. D. Searle & Co., Chicago, Ill.
[8] CPD No. SC-9376, Lot MA8-2604, G. D. Searle & Co., Chicago, Ill.
[9] Merck & Co., West Point, Pa.
[10] Fisher Scientific Co., Pittsburgh, Pa.
[11] Soluene-100 diluted 1:1 in isopropanol or Instagel in 0.5 N HCl 9:1 for scintillation, Packard Instruments Co., Downers Grove, IL 60515
[12] AHR-3050-15: Metabolic Disposition of Bethanidine, A. H. Robins Co., Richmond, Va.
[13] MKI spectrofluorometer–magnetic xenon arc stabilizer, Farrand Optical Co., Valhalla, NY 10595

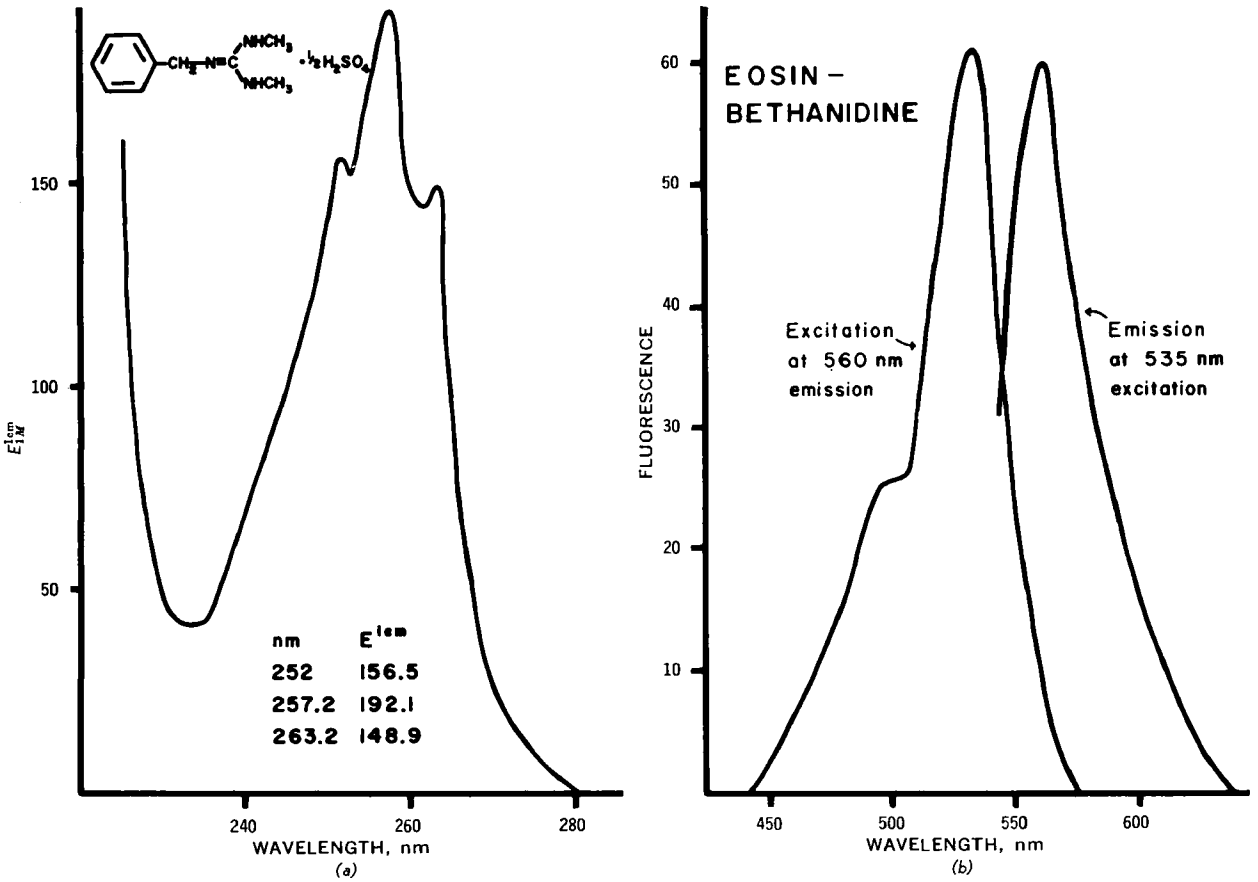

Figure 1—(a) *UV absorption spectrum of bethanidine sulfate in water at 23°.* (b) *Fluorescence excitation and emission spectrum of the bethanidine–eosin complex in chloroform. Bethanidine (0.7 m*M*) in 0.4 m*M *eosin, pH 7.4, was extracted into 5 volumes of chloroform (Steps III and IV).*

tation light path ($t_{1/2}$ = 2 min). The machine is standardized to a preset value before each individual reading with a sealed 1 μM aqueous eosin solution to eliminate small errors due to machine drift. The fluorescence of the eosin reference solution decays with a half-time of approximately 2 weeks.

Concentrations of bethanidine were calculated as follows. The water blank was determined daily (in triplicate) by adding 0.4 ml of borate buffer to 2 ml of water in Step I and assayed to give F_1. The fluorescence equivalent to 1.0 μM bethanidine in water was also determined in triplicate daily by using 0.4 ml of 5 μM bethanidine in borate buffer (diluted from 2.5 mM bethanidine in water stored at 4° for up to 9 months) to give F_2. The patient's prebethanidine plasma blank was determined from the fluorescence (F_3) of borate added to plasma. This plasma blank value was not repeated and was taken to represent the patient's blank throughout therapy (Eq. 1):

$$\text{micromolar in patient blank} = \frac{F_3 - F_1}{F_2 - F_1} \qquad \text{(Eq. 1)}$$

The apparent extraction efficiency (AEE) was determined in triplicate on each patient's prebethanidine plasma:

$$\text{percent } AEE = \left(\frac{F_4 - F_3}{F_2 - F_1}\right) \times 100 \qquad \text{(Eq. 2)}$$

Bethanidine in borate buffer was added to 2 ml of plasma (it is not necessary to preincubate) and assayed to give F_4. The apparent extraction efficiency was calculated and used to correct subsequent analyses on the patient's plasma, if necessary. The apparent extraction efficiency was 95 ± 2% for four patients; if ignored, it would not have much effect on the calculations. The bethanidine levels in the plasma of patients receiving bethanidine were determined in duplicate from the fluorescence (F_5) of plasma added in Step I and calculated from:

$$\text{micromolar} = \left[\left(\frac{F_5 - F_1}{F_2 - F_1}\right) - \text{micromolar in patient blank}\right]\left(\frac{100}{AEE}\right) \qquad \text{(Eq. 3)}$$

It should be kept in mind that due to variable factors that affect fluorescence, the F_1 and F_2 in Eqs. 1 and 2 will be different from the F_1 and F_2 of Eq. 3 unless F_3, F_4, and F_5 are all determined on the same run.

RESULTS

Radioactive ^{14}C-bethanidine (1 μM) in 0.1 N NaOH was extracted with chloroform by shaking the mixture in a stoppered test tube for 5 min. The chloroform layer was taken to dryness under nitrogen, and the radioactivity was determined. An aqueous chloroform ratio of from 1:4 to 1:8 gave a maximal extraction efficiency of 90%. Thus, in Steps I and II, a ratio of 1:5 was selected for the assay. In a second experiment, with this ratio of 1:5 less than 5% of the bethanidine was extracted into aqueous solutions with varying pH of 6–10. Above pH 10, the percentage of bethanidine extraction was maximal by pH 13.5. This finding is compatible with the high pKa of guanidine compounds (3).

In a third series of experiments, plasma containing no bethanidine and with varying pH of 6–13.5 was similarly extracted with chloroform. The chloroform extract was then subjected to the fluorescence development and measurement of Steps III and IV. The plasma blank was maximal below pH 8.0 and was unchanged at pH 9 and above. Thus pH 9.5–10.0 was chosen for Step I to extract maximally fluorescence material in the blank without losing bethanidine; pH 13.5 was chosen to extract maximally bethanidine in Step II.

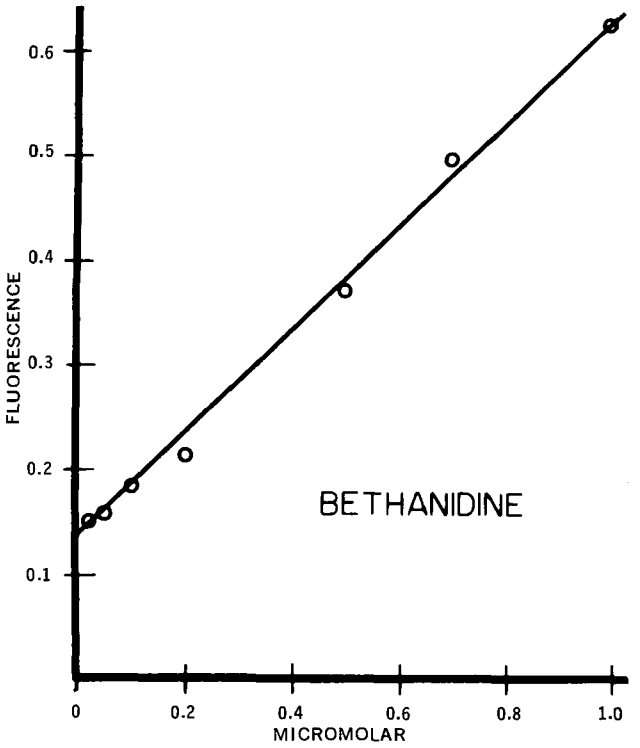

Figure 2—*Linearity of bethanidine–eosin fluorescence. Bethanidine sulfate in water was assayed by the standard procedure. A similar curve was obtained for bethanidine in plasma.*

The UV absorption spectrum exhibited by bethanidine sulfate in water is depicted in Fig. 1*a*. The molar extinction was too low for a sensitive assay utilizing absorption spectroscopy.

The fluorescence of the bethanidine–eosin complex (Fig. 1*b*) provided the necessary sensitivity for a chemically useful assay. Eosin has been complexed previously with alkaloids and quaternary ammonium compounds (4). The bethanidine–eosin complex formed at pH 7.4 in Step III is the most stable when measured fluorometrically in Step IV. The fluorescence is greater up to pH 8.0 and is less below 7.4. The concentration of eosin may be varied, but 4×10^{-4} *M* gives sufficient sensitivity with an acceptable blank.

In performing Steps I–IV, several precautions are necessary for reproducibility. Clean glassware is essential. Eosin may undergo quinoid–lactoid transformation (5). Chloroform is unstable in alkali (6). The fluorescence of the blank increases progressively as contact time with alkaline solutions is prolonged, and at the same time the net fluorescence exhibited by the bethanidine–eosin complex decreases. The mechanism may be due to the degradation

Table I—Relative Fluorescence[a]

Compound	Concentration	Relative Fluorescence
Bethanidine (n = 16)	1 μM	1.00 (0.02)
Guanethidine	1 μM	0.75
Hydrochlorothiazide	10 μM	0
Chlorothiazide	10 μM	0
Benzthiazide	10 μM	0
Urea	40 mM	0
Creatinine	1 mM	0
Uric acid	0.3 mM	0
Spironolactone	1 μM	0
Spironolactone metabolite (dethioacetylated Δ^6-derivative)	1 μM	0
Propranolol	10 μM	0
Aspirin	0.3 mM	0
Fenfluramine	10 μM	0

[a] The compounds were dissolved in water and carried through the standard assay. Relative fluorescence is expressed as the net fluorescence of the test substance in the assay compared to the net fluorescence of 1 μM bethanidine. The fluorescence in parenthesis is the standard deviation.

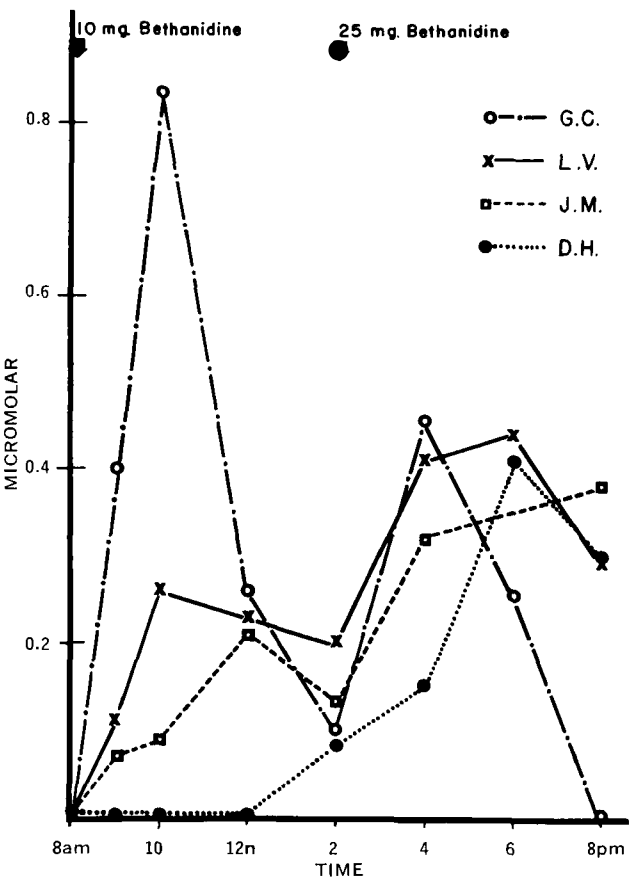

Figure 3—*Bethanidine in plasma. Bethanidine was administered orally to patients at the times indicated (see text).*

products of chloroform, but it has not been determined whether the fluorescence fails to develop or whether instead the developed fluorescence becomes extremely labile on exposure to light. Methylene chloride and ethylene dichloride were not acceptable substitutes for chloroform. The assay is more reproducible when conducted in a dimly lighted room and with aluminum foil-covered tubes.

The fluorescence in Step IV is stable in the dark for about 2 hr, but it is very labile in the excitation light path of 535 nm ($t_{1/2}$ = 2 min); it can be recovered with a $t_{1/2}$ of 20 min by allowing the bethanidine–eosin complex to stand in darkness. If the chloroform layer in Step IV is exposed to air, the fluorescence irreversibly decays ($t_{1/2}$ = 30 min). The 0.75% ethanol used as a stabilizer in the commercially available chloroform is optimal for the assay, and the commercial source is important. Several other sources of chloroform gave high blanks and low net fluorescence. The fluorescence is not affected between 10 and 30°, but at 4° the chloroform becomes supersaturated and aqueous droplets form. Other studies have also revealed a flat temperature coefficient for eosin in water (7). The entire assay should be uniformly timed within and between steps. Usually 16 samples can be analyzed in about 3 hr.

The fluorescence of 0–1 μM bethanidine in water is shown in Fig. 2. It is linear up to 10 μM bethanidine in water or plasma. The small daily variation in fluorescence of the complex is corrected by a standard of bethanidine in each run. The standard deviation of the net fluorescence of bethanidine in a single assay (n = 16) is 2%. The fluorescence of the reagent blank averaged 0.17 ± 0.02 μM, and the plasma blank in six patients was 0.14 ± 0.05 μM bethanidine. One can reproducibly detect about 0.02 μM (4 ng/ml). The assay readily detects guanethidine (Table I); however, the known metabolites of guanethidine have not been subjected to the assay. The fluorescence of some body constituents and drugs was also examined.

To compare the fluorometric assay with the radioactive assay, radioactive ^{14}C-bethanidine was given intravenously (1 μmole/kg) to an 11.2-kg male monkey (*Macaca mulatta*). The solution was standardized by radioactivity and fluorometry. The blood level of

bethanidine at 22 min was 1.01 μM as determined by both assays. Although the radioactive $t_{1/2}$ in plasma was 27 min with a prolonged phase of 4 hr, there was insufficient plasma for the fluorometric assay. Previous reports concerning cats and humans have not revealed any metabolites of bethanidine (8, 9). This matter has not been evaluated in chronic therapy, but the metabolites of bethanidine apparently do not interfere with the assay.

A study on hypertensive patients receiving bethanidine is underway in this laboratory. Bethanidine levels in plasma, correlation with efficacy during acute and chronic therapy, and pharmacokinetic parameters are being determined. Blood levels in four patients who received orally 10 mg followed by 25 mg were variable (Fig. 3). The values demonstrate that the fluorometric assay is readily applicable to analysis of blood levels under clinical conditions. After 3 weeks of therapy at an average dose of 20 mg given at 8 am, 2 pm, and 8 pm, the levels in the four patients were 0.39 ± 0.16 μM. Subsequent studies indicated that the therapeutic range on various dose levels is about 0.3–1.8 μM when determined 2 hr after the afternoon dose of drug[14].

DISCUSSION

This simple fluorometric assay of bethanidine is sufficiently sensitive for application in controlled clinical studies of bethanidine blood levels. Numerous drugs have been complexed previously by similar methods (4, 10). While several other dyes were examined in this study, only eosin Y was acceptable. This assay is not absolutely specific for bethanidine. However, under controlled conditions in which interfering substances (*i.e.*, guanethidine) are not administered and the differential extraction procedure is used, the assay is relatively specific.

Other assays for bethanidine have been described. It has been measured in urine with the alkaline hypobromite reaction (9). The reaction has a sensitivity of about 44 μM bethanidine, in contrast to 0.02 μM for the present fluorometric study. Another method for urine analyses utilized column chromatography followed by complexation of bethanidine with bromcresol green, and it had a limit of sensitivity of approximately 10 μM (11).

An elaborate method for urine and plasma involved a multistep extraction of the drug followed by GC (12). The final analysis was by flame ionization and multiple-ion detection. Bethanidine in plasma was not reported; however, guanethidine in plasma at 0.04 μM (20 ng/ml) gave a standard deviation of 9%. Assuming bethanidine acted similarly in that method, the sensitivity would be in a range approaching the fluorometric method.

Bethanidine is utilized in the therapy of severe hypertension and in acute exacerbations of blood pressure such as may occur in cerebrovascular accidents. The availability of the fluorometric assay described here should be valuable in pharmacokinetic and other clinical studies of this drug. In cases where antihypertensive effects are inadequate, it can be utilized for determining blood levels so that the extent of absorption of the drug or the problem of patient compliance can be examined. Six to eight samples can be analyzed in duplicate in about 3 hr by one person, and a minimum investment in equipment or technical assistance is required.

[14] Unpublished data.

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