

# Manual

Accompanying the GPHF-Minilab™

**Supplement 2023**  
**on more Medicine to Treat**  
**Cardiovascular Disorders**

## Physical Testing & Thin-Layer Chromatography



**Richard W. O. Jähnke and Kornelia Dwornik**



A charity voluntarily supported by  
Merck KGaA, Darmstadt, Germany

# Table of Contents

Chapter	Page
Health & Safety.....	3
New TLC Test Protocols.....	4
7.108 Apixaban.....	4
7.109 Candesartan cilexetil <i>incl. hydrochlorothiazide or amlodipine co-formulations</i> .....	8
7.110 Clopidogrel <i>sulphate/besylate/hydrochloride salt incl. ASA co-formulations and related ticlopidine</i> .....	12
7.111 Hydralazine <i>hydrochloride in oral and parenteral formulations</i> .....	16
7.112 Rivaroxaban .....	20
7.113 Warfarin <i>sodium incl. related phenprocoumon</i> .....	24

# Health & Safety

## Important Notice

The chemicals travelling alongside the GPHF-Minilab™ as well as pharmaceuticals to be tested may contain hazardous substances. Hence, users of the Minilab and bystanders should closely follow all instructions given in this and the main manual in order to avoid potential health risks resulting from accidental contact with these chemical and pharmaceutical substances.

Care must be exercised in the handling of chemicals and pharmaceuticals in order

to avoid generating excessive dust or vapours in the atmosphere. Extraction should be used at points of activity that, in more austere circumstances, might be replaced by simple but sufficient air ventilation.

Symptoms such as drowsiness, respiratory problems, nausea or skin rash must be reported to the supervisor especially after accidental spillage of large amounts of organic solvents.

In the event of accidental spillage or splashing of liquids affecting skin or eyes,

wash with copious amounts of water, report to the supervisor and if necessary, to the local surgery for further attention. Use protective clothes and safety spectacles when handling aggressive test solutions, for example strong acids and caustic solutions.



*Use protective clothing, for example an apron and safety spectacles, prior to starting any work on medicines quality testing. Wash hands and face thoroughly after work.*

## 7.108 Apixaban

### Primary Screening on Product Deficiencies by Physical Testing

#### I. PHYSICAL TESTING

During visual inspection, search for deficiencies on labelling, packaging and dosage forms as described in the opening chapters on general methods and operations of the main manual and report the findings. Consider to take pictures, for example, with a smartphone camera. Each tablet usually contains 2.5 or 5 mg of apixaban. Verify the total weight of tablets and capsules using the electronic pocket balance provided. All quick release

apixaban tablet and capsule formulations have to pass the disintegration test as described at the beginning of the main manual. They should disintegrate in water at 37 °C in less than 30 minutes. It is a major defect if an instant release formulation does not pass this test.

and medicinal products with defective dosage forms, packaging or with incomplete, damaged or missing labels or with labels in a foreign language or medicinal products held under poor storage conditions should be subjected to a thin-layer chromatographic test.

#### II. RESULTS & ACTIONS TO BE TAKEN

Medicinal products from unusually cheap sources, medicinal products with missing or incorrect accompanying documents

### Verification of Drug Identity and Content by Thin-Layer Chromatography

#### I. PRINCIPLE

Apixaban is extracted from tablets or capsules with a known volume of concentrated acetic acid solution and is subsequently checked for identity and content by thin-layer chromatography (TLC) in comparison with a suitable secondary standard.

#### II. EQUIPMENT AND REAGENTS

- 1) Pestle
- 2) Aluminium foil
- 3) Funnel
- 4) Spatula
- 5) Label tape
- 6) Marker pen
- 7) Pencil and ruler
- 8) 10-ml vials
- 9) Set of graduated pipettes (1 to 25 ml)
- 10) Set of laboratory glass bottles (25 to 100 ml)
- 11) Merck TLC aluminium plates pre-coated with silica gel 60 F<sub>254</sub>, size 5x10 cm
- 12) Glass microcapillaries (2-µl filling capacity)
- 13) TLC developing chamber (500-ml jar)
- 14) Hot plate
- 15) Filter paper
- 16) Pair of scissors
- 17) Pair of tweezers
- 18) UV light of 254 nm
- 19) Methanol
- 20) Ethyl acetate
- 21) Acetic acid solution 96%
- 22) Reference agent, for example, apixaban 5 mg tablets

### III. PREPARATION OF THE STOCK STANDARD SOLUTION

The preparation of the stock standard solution requires an authentic medicinal product for reference purposes, for example, tablets containing 5 mg of apixaban. Wrap up one reference tablet into aluminium foil and crush it down to a fine powder using a pestle. Carefully empty the aluminium foil over a 25-ml laboratory glass bottle and wash down all residual solids with 10 ml of acetic acid solution 96% using a graduated pipette. Close the bottle and shake for about three minutes. Filter the suspension obtained through the filter paper provided and collect the filtrate in a 10-ml vial. It takes about 5 minutes to collect approximately 6 ml of a hazy filtrate. The hazy solution obtained should contain 0.5 mg of total apixaban per ml and be labelled as '*Apixaban Stock Standard Solution*'. Freshly prepare this solution for each test. Continue working with the hazy liquid obtained.

### IV. PREPARATION OF THE WORKING STANDARD SOLUTION 100% (UPPER WORKING LIMIT)

The stock standard solution requires no further dilution. It already represents the final working concentration of 0.5 mg of total apixaban per ml.

This higher working standard solution represents a medicinal product of good quality containing 100% of apixaban.

### V. PREPARATION OF THE WORKING STANDARD SOLUTION 80% (LOWER WORKING LIMIT)

Pipette 4 ml of the stock standard solution into a 10-ml vial and add 1 ml of acetic acid solution 96% using suitable graduated pipettes. Close and shake the vial. The solution obtained should contain 0.4 mg of total apixaban per ml and be labelled as '*Apixaban Working Standard Solution 80%*'.

This lower working standard solution represents a medicinal product of poor quality containing just 80% of the amount of apixaban as stated on the product's label. In the current investigation, this level of apixaban represents the lower acceptable limit for a given product. Pharmacopoeial limits do not apply in our context.

### VI. PREPARATION OF THE STOCK SAMPLE SOLUTION FROM A PRODUCT CLAIMING TO CONTAIN 2.5 MG OF APIXABAN PER UNIT

Take two whole tablets or capsules from a suitable medicinal product sampled in the field. As usual, tablets are wrapped up in aluminium foil and crushed down to a fine powder. Transfer all the powder obtained into a 25-ml laboratory glass bottle. Powder obtained from sample capsules should be placed directly in the bottle adding the empty cap and body shells last. For extraction, add 10 ml of acetic acid solution 96% using a suitable graduated pipette. Close the bottle and shake for about three minutes. If the powder forms a sort of stable suspension, then filter the liquid through the filter paper provided and collect the filtrate in a 10-ml vial. After about 5 minutes, approximately 6 ml of a hazy stock solution are collected. This solution is far away from perfect but can be used for spotting already. If the powder forms a slightly turbid or even clear solution only, then do not filter and just let the solution stand for another five minutes until undissolved residues settle under the supernatant liquid.

**Note:** If no filtration is required, one sample tablet/capsule would be enough to produce the stock sample solution using only 5 ml of acetic acid solution 96% for extraction.

#### 5 MG OF APIXABAN PER UNIT

Place the powder obtained from a whole sample tablet or capsule into a 25-ml laboratory glass bottle, add 10 ml of acetic acid solution 96% with a suitable graduated pipette and extract the apixaban. Continue working as described above. Filtration may be required.

All stock sample solutions produced should finally contain 0.5 mg of total apixaban per ml and be labelled as '*Apixaban Stock Sample Solution*'. Freshly prepare these solutions for each test. Continue working with the hazy or clear liquid obtained.

## VII. PREPARATION OF THE WORKING SAMPLE SOLUTION

The stock sample solutions require no further dilution. They already represent the final working concentration of 0.5 mg of apixaban per ml. If prepared from a high quality product, the sample solution should match the concentration of apixaban of the higher working standard solution produced above.

## VIII. SPOTTING

Mark an origin line parallel to and about 1.5 cm from the bottom edge of the chromatoplate and apply 2 µl of each test and standard solution as shown in the picture opposite using the microcapillary pipettes supplied.

Up to five spots can be placed on a plate. Check the uniformity of all spots using UV light of 254 nm. All spots should be circular in shape and equally spaced across the origin line. Although their intensities might differ, their diameters never should. Different intensities are due to residual amounts of excipients or different drug concentrations in the sample solutions. A difference in spot size, however, relates to poor spotting. Repeat this step if homogeneous spotting is not achieved first time.

Gently dry the spots. To do this, hold the chromatoplate with the supplied tweezers in the hot air stream directly above the heating plate for about one minute. Shake the TLC plate constantly and each time the chromatoplate moves downwards, its underside is allowed to touch the surface of the heating plate for fractions of a second.

## IX. DEVELOPMENT

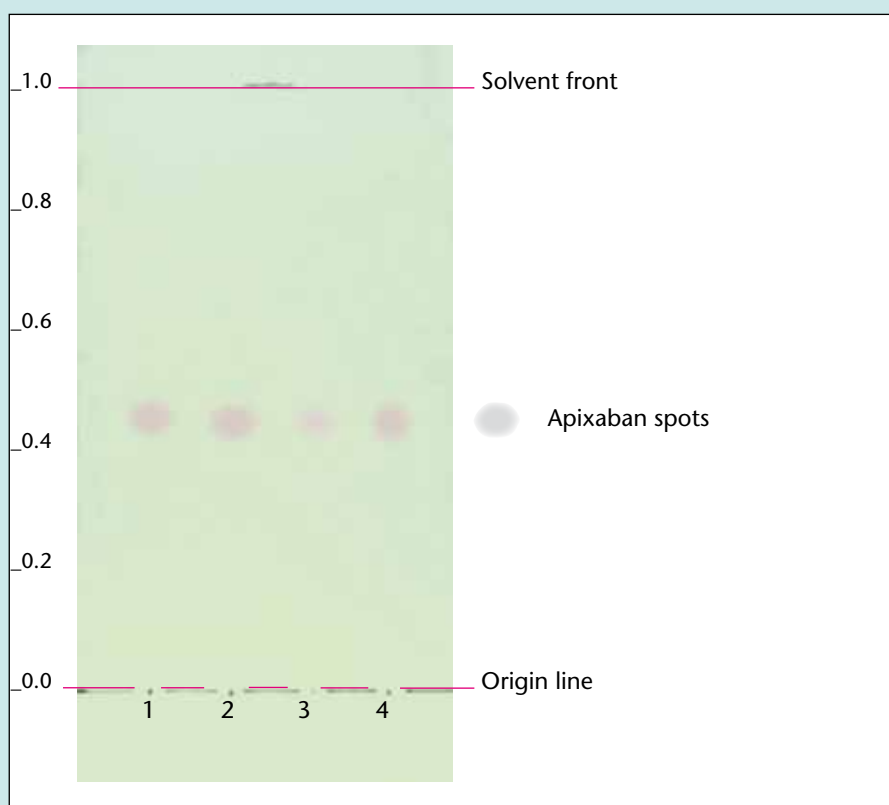
Using suitable graduated pipettes, add 15 ml of ethyl acetate, 5 ml of methanol and 0.5 ml of acetic acid solution 96% to the jar serving as TLC developing chamber. Close the chamber and mix thoroughly. Line the chamber's wall with filter paper and wait for about 15 minutes thus ensuring saturation of the chamber with solvent vapour. Carefully place the loaded TLC plate into the jar. Close the jar and develop the chromatoplate until the solvent front has moved about three-quarters of the length of the plate, the developing time being about 15 minutes. Remove the TLC plate from the chamber, mark the solvent front and allow excess solvent to evaporate by gentle drying. To do this, hold the chromatoplate with the supplied tweezers in the hot air stream directly above the heating plate for about two minutes. Shake the TLC plate constantly and each time the chromatoplate moves downwards, its underside is allowed to touch the surface of the heating plate for fractions of a second.

## X. DETECTION

After drying off all solvent residues, view the chromatography plate under UV light at 254 nm with the battery driven lamp provided. Use this method of detection of both, identification and quantification of apixaban.

CHROMATOPLATE OBSERVED UNDER  
UV LIGHT OF 254 NM

- Run No.1:  
Upper working standard representing  
100% of total apixaban
- Run No.2:  
A product of good quality with acceptable  
apixaban content
- Run No.3:  
A product of poor quality with unaccept-  
able low apixaban content
- Run No.4:  
Lower working standard representing  
80% of total apixaban



XI. OBSERVATIONS MADE AT 254 NM

A dark spot at a travel distance of about 0.45 indicates the presence of apixaban in the test solution. Additional strong spots generated by the test solution would point at other drugs or apixaban degradation, the latter case being more likely when associated with a smaller principal spot. A smaller principal spot from the test solution may also indicate a poor apixaban content and no spot at all a complete absence of apixaban. Related active agents, for example rivaroxaban, would show a relative retention factor of about 0.53. Auxiliary agents incorporated in different finished products might cause some fainter spots either travelling up to the solvent front or emerging near or on the origin line. Lactose remains invisible but additional staining tests with sulphuric acid and heat indicate that it would show up at about 0.10 if present in the formulation.

XII. RESULTS & ACTIONS TO BE TAKEN

The apixaban spot in the chromatogram obtained with the test solution must correspond in terms of colour, size, intensity, shape and travel distance to that in the chromatogram obtained with the lower and higher standard solution. This result must be obtained for each method of detection. If this is not achieved, repeat the run from scratch with a second sample. Reject the batch if the drug content cannot be verified in a third run. For a second opinion, refer additional samples to a fully-fledged drug quality control laboratory. Retain samples and put the batch on quarantine until a final decision on rejection or release has been taken. For documentation purposes, take pictures of all the readings with a digital camera turning off the flash first.

## 7.109 Candesartan cilexetil incl. hydrochlorothiazide or amlodipine co-formulations

### Primary Screening on Product Deficiencies by Physical Testing

#### I. PHYSICAL TESTING

During visual inspection, search for deficiencies on labelling, packaging and dosage forms as described in the opening chapters on general methods and operations of the main manual and report the findings. Consider to take pictures, for example, with a smartphone camera. Each tablet usually contains 4, 8, 16 or 32 mg of candesartan cilexetil ester. Tablets and capsules may be combined with 12.5 or

25 mg of hydrochlorothiazide or with 5 or 10 mg of amlodipine. Verify the total weight of tablets and capsules using the electronic pocket balance provided. All quick release candesartan tablet or capsule formulations have to pass the disintegration test as described in the main manual. They should disintegrate in water at 37 °C in less than 30 minutes. It is a major defect if an instant release formulation does not pass this test.

#### II. RESULTS & ACTIONS TO BE TAKEN

Medicinal products from unusually cheap sources, medicinal products with missing or incorrect accompanying documents and medicinal products with defective dosage forms, packaging or with incomplete, damaged or missing labels or with labels in a foreign language or medicinal products held under poor storage conditions should be subjected to a thin-layer chromatographic test.

### Verification of Drug Identity and Content by Thin-Layer Chromatography

#### I. PRINCIPLE

Whether or not combined with other cardiac medicines, candesartan cilexetil ester is extracted from tablets or capsules with a known volume of acetone and is then checked for identity and content by thin-layer chromatography (TLC) in comparison to a suitable secondary standard. For fixed-dose combinations, refer to the hydrochlorothiazide or amlodipine protocol in the main manual for additional testing.

#### II. EQUIPMENT AND REAGENTS

- 1) Pestle
- 2) Aluminium foil
- 3) Funnel
- 4) Spatula
- 5) Label tape
- 6) Marker pen
- 7) Pencil and ruler
- 8) 10-ml vials
- 9) Set of graduated pipettes (1 to 25 ml)
- 10) Set of laboratory glass bottles (25 to 100 ml)
- 11) Merck TLC aluminium plates pre-coated with silica gel 60 F<sub>254</sub>, size 5x10 cm
- 12) Glass microcapillaries (2-µl filling capacity)
- 13) TLC developing chamber (500-ml jar)
- 14) Hot plate
- 15) Filter paper
- 16) Pair of scissors
- 17) Pair of tweezers
- 18) UV light of 254 nm
- 19) UV light of 366 nm
- 20) Methanol
- 21) Acetone
- 22) Ethyl acetate
- 23) Acetic acid solution 96%
- 24) Reference agent, for example, candesartan cilexetil 8 mg tablets



### III. PREPARATION OF THE STOCK STANDARD SOLUTION

The preparation of the stock standard solution requires an authentic medicinal product for reference purposes, for example, tablets containing 8 mg of candesartan cilexetil ester. Wrap up one reference tablet into aluminium foil and crush it down to a fine powder using a pestle. Carefully empty the aluminium foil over a 10-ml laboratory glass bottle and wash down all residual solids with 4 ml of acetone using a graduated pipette. Close the lab bottle and shake for about three minutes until most of the solids are dissolved. Allow the solution to stand for an additional five minutes until undissolved residues settle below the supernatant liquid. The solution obtained should contain 2 mg of total candesartan cilexetil per ml and be labelled as '*Candesartan Stock Standard Solution*'. Freshly prepare this solution for each test. Continue working with the clear or hazy supernatant liquid.

### IV. PREPARATION OF THE WORKING STANDARD SOLUTION 100% (UPPER WORKING LIMIT)

Pipette 1 ml of the stock standard solution into a 10-ml vial and add 3 ml of acetone using suitable graduated pipettes. Close and shake the vial. The solution obtained should contain 0.5 mg of total drug per ml and be labelled as '*Candesartan Working Standard Solution 100%*'.

This higher working standard solution represents a medicinal product of good quality containing 100% of candesartan cilexetil ester.

### V. PREPARATION OF THE WORKING STANDARD SOLUTION 80% (LOWER WORKING LIMIT)

Pipette 1 ml of the stock standard solution into a 10-ml vial and add 4 ml of acetone using suitable graduated pipettes. Close and shake the vial. The solution obtained should contain 0.4 mg of total drug per ml and be labelled as '*Candesartan Working Standard Solution 80%*'.

This lower working standard solution represents a medicinal product of poor quality containing just 80% of the amount of candesartan cilexetil ester as stated on the product's label. In the current investigation, this level of candesartan cilexetil represents the lower acceptable limit for a given product. Pharmacopoeial limits do not apply in our context.

### VI. PREPARATION OF THE STOCK SAMPLE SOLUTION FROM A PRODUCT CLAIMING TO CONTAIN 4 MG OF CANDESARTAN CILEXETIL PER UNIT

Take one whole tablet or capsule from a suitable medicinal product sampled in the field. As usual, tablets are wrapped up in aluminium foil and crushed down to a fine powder. Transfer all the powder obtained into a 10-ml laboratory glass bottle. Powder obtained from a sample capsule should be placed directly in the bottle adding the empty cap and body shells last. For extraction, add 2 ml of acetone using a suitable graduated pipette. Then, close the bottle and shake for about three minutes until most of the solids are dissolved. Allow the solution to stand for an additional five minutes until undissolved residues settle below the supernatant liquid.

#### 8 MG OF CANDESARTAN CILEXETIL PER UNIT

Place the powder obtained from a whole sample tablet or capsule in a 10-ml laboratory glass bottle, add 4 ml of acetone with a suitable graduated pipette and extract the candesartan cilexetil ester. Continue working as described above.

#### 16 MG OF CANDESARTAN CILEXETIL PER UNIT

Place the powder obtained from a whole sample tablet or capsule in a 10-ml laboratory glass bottle, add 8 ml of acetone with a suitable graduated pipette and extract the candesartan cilexetil ester. Continue working as described above.

#### 32 MG OF CANDESARTAN CILEXETIL PER UNIT

Place the powder obtained from a whole sample tablet or capsule in a 25-ml laboratory glass bottle, add 16 ml of acetone with a suitable graduated pipette and extract the candesartan cilexetil ester. Continue working as described above.

Whether or not combined with other cardiac medicines, all stock sample solutions produced should finally contain 2 mg of total candesartan cilexetil ester per ml and be labelled as '*Candesartan Stock Sample Solution*'. Freshly prepare these solutions for each test. Continue working with the clear or hazy supernatant liquids.

## VII. PREPARATION OF THE WORKING SAMPLE SOLUTION

Pipette 1 ml of the stock sample solution into a 10-ml vial and add 3 ml of acetone. Close and shake the vial and label as 'Candesartan Working Sample Solution'.

The expected concentration of candesartan cilexetil ester in the working sample solutions is 0.5 mg per ml and should correspond to the concentration of candesartan cilexetil in the higher working standard solution prepared above.

## VIII. SPOTTING

Mark an origin line parallel to and about 1.5 cm from the bottom edge of the chromatoplate and apply 2 µl of each test and standard solution as shown in the picture opposite using the microcapillary pipettes supplied.

Up to five spots can be placed on a plate. Check the uniformity of all spots using UV light of 254 nm. All spots should be circular in shape and equally spaced across the origin line. Although their intensities might differ, their diameters never should. Different intensities are due to residual amounts of excipients or different drug concentrations or combinations in the sample solutions. A difference in spot size, however, relates to poor spotting. Repeat this step if homogeneous spotting is not achieved first time.

Allow the spots to air-dry for about three minutes until the smell of acetone is almost completely gone. Avoid any heat drying.

## IX. DEVELOPMENT

Using suitable graduated pipettes, add 18 ml of ethyl acetate, 2 ml of methanol, and 0.1 ml of acetic acid solution 96% to the jar serving as TLC developing chamber. Close the chamber and mix thoroughly. Line the chamber's wall with filter paper and wait for about 15 minutes thus ensuring saturation of the chamber with solvent vapour. Carefully place the loaded TLC plate into the jar. Close the jar and develop the chromatoplate until the solvent front has moved about three-quarters of the length of the plate, the developing time being about 10 minutes. Remove the TLC plate from the chamber, mark the solvent front and allow excess solvent to evaporate. To do this, allow the spots to air-dry for about five minutes. Avoid any heat drying.

## X. DETECTION

After drying off all solvent residues, view the chromatography plate under UV light at 254 nm with the battery-powered lamp provided. Use this detection method for the identification and quantification of candesartan cilexetil. If combined with hydrochlorothiazide (HCT), the spot for this agent will appear above the candesartan cilexetil spot. If combined with amlodipine, an additional spot can be detected very near or even on the line of origin. When the latter spot is irradiated with UV light at 366 nm, a strong white fluorescence appears. For checking the HCT or amlodipine content, refer to the relevant assay protocols in the main manual.

## XI. OBSERVATIONS MADE AT 254 NM

A strong blue-violet spot at a travel distance of about 0.42 indicates the presence of candesartan cilexetil in the test solution. Additional strong spots generated by the test solution would point at other drugs or candesartan cilexetil degradation, the latter case being more likely when associated with a smaller principal spot. A smaller princi-

CHROMATOPLATE OBSERVED UNDER  
UV LIGHT OF 254 NM

Run No.1:

Upper working standard representing  
100% of total candesartan cilexetil

Run No.2:

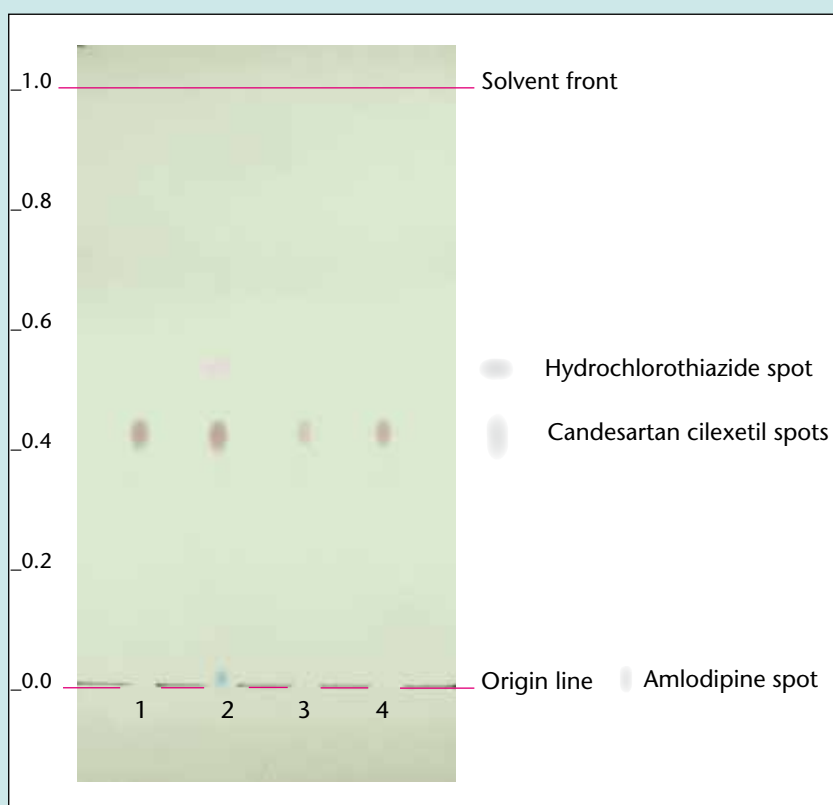
A fixed-dose combination product of  
good quality with acceptable content of  
candesartan cilexetil

Run No.3:

A product of poor quality with unaccept-  
able low candesartan cilexetil content

Run No.4:

Lower working standard representing  
80% of total candesartan cilexetil



pal spot from the test solution may also indicate a poor candesartan cilexetil content and an absent spot may indicate a complete absence of candesartan. If candesartan cilexetil is combined with hydrochlorothiazide (HCT), a second spot can be observed at a travel distance of about 0.54 above the spot for candesartan. If candesartan cilexetil is combined with amlodipine, an additional spot is visible at a travel distance of about 0.02 near the line of origin. Excipients present in various finished products may cause fainter spots that either migrate up to the solvent front or linger near or on the line of origin.

XII. OBSERVATIONS MADE AT 366 NM

When candesartan cilexetil is combined with amlodipine, the presence of the latter compound is confirmed by a strong white fluorescence at a travel distance of about 0.02 very close to the origin line.

XIII. RESULTS & ACTIONS TO BE TAKEN

The candesartan cilexetil spot in the chromatogram obtained with the test solution must correspond in terms of colour, size, intensity, shape and travel distance to that in the chromatogram obtained with the lower and higher standard solution. This result must be obtained for each method of detection. If this is not achieved, repeat the run from scratch with a second sample. Reject the batch if the drug content cannot be verified in a third run. For a second opinion, refer additional samples to a fully-fledged drug quality control laboratory. Retain samples and put the batch on quarantine until a final decision on rejection or release has been taken. For documentation purposes, take pictures of all the readings with a digital camera turning off the flash first.

## 7.110 Clopidogrel sulphate/besylate/hydrochloride salt incl. ASA co-formulations

### Primary Screening on Product Deficiencies by Physical Testing

#### I. PHYSICAL TESTING

During visual inspection, search for deficiencies on labelling, packaging and dosage forms as described in the opening chapters on general methods and operations of the main manual and report the findings. Consider to take pictures, for example, with a smartphone camera. Whether clopidogrel comes as a sulphate, besylate or hydrochloride salt, each tablet usually contains 75 mg of clopidogrel per

free base. Other dosage strengths and fixed-dose combinations with acetylsalicylic acid (ASA) are known to exist. Verify the total weight of tablets and capsules using the electronic pocket balance provided. All quick release clopidogrel tablet and capsule formulations have to pass the disintegration test as described at the beginning of the main manual. They should disintegrate in water at 37 °C in less than 30 minutes. It is a major defect if an instant release formulation does not pass this test.

#### II. RESULTS & ACTIONS TO BE TAKEN

Medicinal products from unusually cheap sources, medicinal products with missing or incorrect accompanying documents and medicinal products with defective dosage forms, packaging or with incomplete, damaged or missing labels or with labels in a foreign language or medicinal products held under poor storage conditions should be subjected to a thin-layer chromatography test.

### Verification of Drug Identity and Content by Thin-Layer Chromatography

#### I. PRINCIPLE

Clopidogrel sulphate/besylate/hydrochloride is extracted from tablets or capsules with a known volume of methanol and is subsequently checked for identity and content by thin-layer chromatography (TLC) in comparison with a suitable secondary standard. For fixed combinations with ASA, refer to the acetylsalicylic acid protocol in the main manual for additional testing. Incidentally, the same mobile phase is used for both, the clopidogrel and the acetylsalicylic acid test protocol.

#### II. EQUIPMENT AND REAGENTS

- |   |  |
|---|--|
| 1) Pestle   | 14) Hot plate  |
| 2) Aluminium foil   | 15) Filter paper   |
| 3) Funnel   | 16) Pair of scissors   |
| 4) Spatula  | 17) Pair of tweezers   |
| 5) Label tape   | 18) UV light of 254 nm   |
| 6) Marker pen   | 19) UV light of 366 nm   |
| 7) Pencil and ruler   | 20) Iodine chamber   |
| 8) 10-ml vials  | 21) Methanol   |
| 9) Set of graduated pipettes<br>(1 to 25 ml)  | 22) n-Butanol  |
| 10) Set of laboratory glass bottles<br>(25 to 100 ml)   | 23) Ethyl acetate  |
| 11) Merck TLC aluminium plates<br>pre-coated with silica gel 60 F <sub>254</sub> <sup>r</sup><br>size 5x10 cm | 24) Acetic acid solution 96%   |
| 12) Glass microcapillaries<br>(2-µl filling capacity)   | 25) Sulphuric acid solution 96%  |
| 13) TLC developing chamber<br>(500-ml jar)  | 26) Distilled/tap/bottled water  |
|   | 27) Reference agent, for example, tablets containing 75 mg of clopidogrel in the form of its sulphate salt |

#### III. PREPARATION OF THE STOCK STANDARD SOLUTION

The preparation of the stock standard solution requires an authentic medicinal product for reference purposes, for example, tablets containing 75 mg of clopidogrel in the form of its sulphate salt. Wrap up one reference tablet into aluminium foil and crush it down to a fine powder using a pestle. Carefully empty the aluminium foil over a 25-ml laboratory glass bottle and wash down all residual solids with 15 ml of methanol using a graduated pipette. Close the lab bottle and shake for about three minutes until most of the solids are dissolved. Allow the solution to stand for an additional five minutes

until undissolved residues settle below the supernatant liquid. The solution obtained should contain 5 mg of total clopidogrel per ml and be labelled as '*Clopidogrel Stock Standard Solution*'. Freshly prepare this solution for each test. Continue working with the clear or hazy supernatant liquid.

#### IV. PREPARATION OF THE WORKING STANDARD SOLUTION 100% (UPPER WORKING LIMIT)

The stock standard solution requires no further dilution. It already represents the final working concentration of 5 mg of total clopidogrel per ml. For more convenient handling, some of the supernatant liquid may be transferred to a new 10-ml vial.

This higher working standard solution represents a medicinal product of good quality containing 100% of clopidogrel per free base (pfb).

#### V. PREPARATION OF THE WORKING STANDARD SOLUTION 80% (LOWER WORKING LIMIT)

Pipette 4 ml of the stock standard solution into a 10-ml vial and add 1 ml of methanol using suitable graduated pipettes. Close and shake the vial. The solution obtained should contain 4 mg of total clopidogrel per ml and be labelled as '*Clopidogrel Working Standard Solution 80%*'.

This lower working standard solution represents a medicinal product of poor quality containing just 80% of the amount of clopidogrel as stated on the product's label. In the current investigation, this level of clopidogrel free base represents the lower acceptable limit for a given product. Pharmacopoeial limits do not apply in our context.

#### VI. PREPARATION OF THE STOCK SAMPLE SOLUTION FROM A PRODUCT CLAIMING TO CONTAIN 75 MG OF CLOPIDOGREL PER UNIT

##### 300 MG OF CLOPIDOGREL PER UNIT

Take a whole tablet or capsule from a suitable medicinal product sampled in the field. As usual, tablets are wrapped up in aluminium foil and crushed down to a fine powder. Transfer all the powder obtained into a 25-ml laboratory glass bottle. Powder obtained from sample capsules should be placed directly in the bottle adding the empty cap and body shells last. For extraction, add 15 ml of methanol using a suitable graduated pipette. Then, close the bottle and shake for about three minutes until most of the solids are dissolved. Allow the solution to stand for an additional five minutes until undissolved residues settle below the supernatant liquid.

Place the powder obtained from a whole sample tablet or capsule into a 100-ml laboratory glass bottle, add 60 ml of methanol with a suitable graduated pipette and extract the clopidogrel. Continue working as described above.

Whether or not combined with ASA, all stock sample solutions produced should finally contain 5 mg of total clopidogrel per ml and be labelled as '*Clopidogrel Stock Sample Solution*'. Freshly prepare these solutions for each test. Continue working with the clear or hazy supernatant liquids.

#### VII. PREPARATION OF THE WORKING SAMPLE SOLUTION

The stock sample solutions require no further dilution. They already represent the final working concentration of 5 mg of clopidogrel per ml. If prepared from a high quality product, the sample solutions should match the concentration of clopidogrel of the higher working standard solution produced above. To facilitate handling, some of the supernatant liquid may be transferred to a 10-ml vial.

#### VIII. SPOTTING

Mark an origin line parallel to and about 1.5 cm from the bottom edge of the chromatoplate and apply 2 µl of each test and standard solution as shown in the picture on page 15 using the microcapillary pipettes supplied.

Up to five spots can be placed on a plate. Check the uniformity of all spots using UV light of 254 nm. All spots should be circular in shape and equally spaced across the origin line. Although their intensities might differ, their diameters never should. Different intensities are due to residual amounts of excipients or different drug concentrations or combinations in the sample solutions. A difference in spot size, however, relates to poor spotting. Repeat this step if homogeneous spotting is not achieved first time.

Allow the spots to air-dry for about two to three minutes until the methanol has almost completely disappeared.

## IX. DEVELOPMENT

A) Mobile phase for clopidogrel content verification: Using suitable graduated pipettes, add 18 ml of ethyl acetate, 4 ml of Methanol and 0.1 ml of acetic acid solution 96% to the jar serving as TLC developing chamber. Close the chamber and mix thoroughly. Line the chamber's wall with filter paper and wait for about 15 minutes thus ensuring saturation of the chamber with solvent vapour. Carefully place the loaded TLC plate into the jar. Close the jar and develop the chromatoplate until the solvent front has moved about three-quarters of the length of the plate, the developing time being about 10 minutes. Remove the TLC plate from the chamber, mark the solvent front and allow excess solvent to evaporate by gentle drying. To do this, hold the chromatoplate with the supplied tweezers in the hot air stream directly above the heating plate for about two minutes. Shake the TLC plate constantly and each time the chromatoplate moves downwards, its underside is allowed to touch the surface of the heating plate for fractions of a second.

B) Mobile phase to distinguish clopidogrel from closely related ticlopidine: Pipette 12 ml of n-butanol, 3 ml of acetic acid solution 96% and 6 ml of water into the TLC developing chamber in the order indicated. Carefully place the loaded TLC plate into the jar saturated with solvent vapour. Close the jar and develop the chromatoplate until the solvent front has moved about half of the length of the plate, the developing time being about 30 minutes. Remove the TLC plate from the chamber, mark the solvent front and allow excess solvent to evaporate by gentle drying in the hot air stream above the heating plate for about three minutes as already outlined above for mobile phase «A».

## X. DETECTION

After drying off all solvent residues, view the chromatographic plate under UV light at 254 nm with the battery driven lamp provided. For additional identification and quantification of clopidogrel, stain the chromatoplate with iodine vapours.

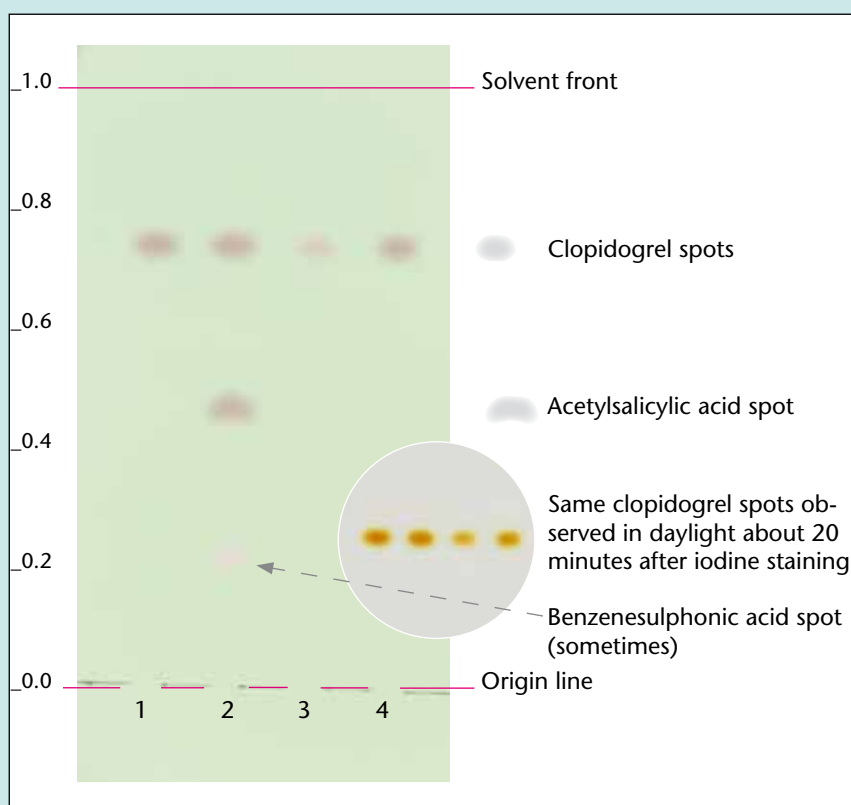
To further distinguish clopidogrel from the closely related ticlopidine, treat the iodine plate with sulphuric acid and heat. To do this, fill the 250 ml plastic beaker provided with 190 ml of methanol, followed by 10 ml of sulphuric acid solution 96%, and mix thoroughly. Allow the mixture to cool and immerse the chromatography plate in the staining solution with the top side down if it is from mobile phase «A» and with the bottom side first if it is from mobile phase «B». Immediately remove the plate from the solution and allow excess liquid to drain onto a paper towel. Wipe the remaining liquid from the back of the plate and dry the entire staining solution for about 30 to 60 seconds at maximum heat setting on the hot plate provided. After removing the chromatographic plate from the heating plate, view the stained plate under UV light at 366 nm in the dark. The spots representing clopidogrel show white fluorescence. The closely related ticlopidine does not show this fluorescence.

## XI. OBSERVATIONS MADE AT 254 NM

Mobile phase A: A dark spot at a travel distance of about 0.73 indicates the presence of clopidogrel in the test solution. Additional strong spots generated by the test solution would point at other drugs or clopidogrel degradation, the latter case being more likely when associated with a smaller principal spot. A smaller principal spot from the test solution may also indicate a poor clopidogrel content and no spot at all a complete absence of clopidogrel. If combined with acetylsalicylic acid (ASA), this compound is visible a travel distance of about 0.47. In addition, benzenesulphonic acid from

CHROMATOPLATE FROM MOBILE  
PHASE «A» OBSERVED UNDER UV  
LIGHT OF 254 NM

- Run No.1:  
Upper working standard representing  
100% of total clopidogrel
- Run No.2:  
A fixed-dose combination product of  
good quality with acceptable clopidogrel  
content
- Run No.3:  
A product of poor quality with unaccept-  
able low clopidogrel content
- Run No.4:  
Lower working standard representing  
80% of total clopidogrel



the clopidogrel besylate salt may become visible at a travel distance of about 0.20. Auxiliary agents incorporated in different finished products might cause some fainter spots either travelling up to the solvent front or emerging near or on the origin line.

Mobile phase B: A dark spot at a travel distance of about 0.90 indicates the presence of clopidogrel in the test solution. Closely related ticlopidine from the same thienopyridine family would show a relative retention factor of about 0.63. The relative retention factor for ASA is about 0.80 and the one for benzenesulphonic acid about 0.52.

XII. OBSERVATIONS MADE IN DAY-  
LIGHT AFTER IODINE STAINING

When the TLC plate is further exposed to iodine vapour, all the clopidogrel spots previously observed at 254 nm now turn yellowish brown. The staining is very strong, making semi-quantitative readings difficult. After a waiting period of about 20 minutes, when the iodine evaporates and the staining matures, different shades and sizes roughly indicate different clopidogrel concentrations. Ticlopidine behaves very similarly here.

XIII. OBSERVATIONS MADE AT 366 NM  
AFTER STAINING THE IODINE PLATE  
WITH SULPHURIC ACID

When the iodine-stained TLC plate is additionally exposed to sulphuric acid and heat, all clopidogrel spots show a white fluorescence in the dark. The closely related ticlopidine does not show this fluorescence. The fluorescence is strongest in the presence of iodine after iodine staining and only shows up at ambient temperature after the TLC plate is removed from the hot plate.

XIV. RESULTS & ACTIONS TO BE TAKEN

The clopidogrel spot in the chromatogram obtained with the test solution must correspond in terms of colour, size, intensity, shape and travel distance to that in the chromatogram obtained with the lower and higher standard solution. This result must be obtained for each method of detection. If this is not achieved, repeat the run from scratch with a second sample. Reject the batch if the drug content cannot be verified in a third run. For a second opinion, refer additional samples to a fully-fledged drug quality control laboratory. Retain samples and put the batch on quarantine until a final decision on rejection or release has been taken. For documentation purposes, take pictures of all the readings with a digital camera turning off the flash first.



## 7.111 Hydralazine hydrochloride in oral and parenteral formulations

### Primary Screening on Product Deficiencies by Physical Testing

#### I. PHYSICAL TESTING

During visual inspection, search for deficiencies on labelling, packaging and dosage forms as described in the opening chapters on general methods and operations of the main manual and report the findings. Consider to take pictures, for example, with a smartphone camera. Liquid dosage forms for parenteral use contain 20 mg of hydralazine hydrochloride salt per unit. Parenteral solutions should be clear and free of foreign matters. In

contrast, tablets usually contain 25, 50 or 100 mg of hydralazine hydrochloride salt. Other dosage strengths are known to exist. Verify the total weight of tablets and capsules using the electronic pocket balance provided. All quick release hydralazine tablet and capsule formulations have to pass the disintegration test as described at the beginning of the main manual. They should disintegrate in water at 37 °C in less than 30 minutes. It is a major defect if an instant release formulation does not pass this test.

#### II. RESULTS & ACTIONS TO BE TAKEN

Medicinal products from unusually cheap sources, medicinal products with missing or incorrect accompanying documents and medicinal products with defective dosage forms, packaging or with incomplete, damaged or missing labels or with labels in a foreign language or medicinal products held under poor storage conditions should be subjected to a thin-layer chromatographic test.

### Verification of Drug Identity and Content by Thin-Layer Chromatography

#### I. PRINCIPLE

Hydralazine hydrochloride solutions are diluted and solid dosage forms extracted with a known volume of water and the test solutions obtained subsequently checked for identity and content by thin-layer chromatography (TLC) in comparison with a suitable secondary standard.

#### II. EQUIPMENT AND REAGENTS

- |  |  |
|--|--|
| 1) Pestle  | 14) Hot plate  |
| 2) Aluminium foil  | 15) Filter paper   |
| 3) Funnel  | 16) Pair of scissors   |
| 4) Spatula   | 17) Pair of tweezers   |
| 5) Label tape  | 18) UV light of 254 nm   |
| 6) Marker pen  | 19) Ninhydrin  |
| 7) Pencil and ruler  | 20) Methanol   |
| 8) 10-ml vials   | 21) Acetone  |
| 9) Set of graduated pipettes<br>(1 to 25 ml)   | 22) Ammonia solution 25%   |
| 10) Set of laboratory glass bottles<br>(25 to 100 ml)  | 23) Distilled/tap/bottled water  |
| 11) Merck TLC aluminium plates<br>pre-coated with silica gel 60 F <sub>254</sub><br>size 5x10 cm | 24) Acetic acid solution 96%   |
| 12) Glass microcapillaries<br>(2-µl filling capacity)  | 25) Electronic pocket balance  |
| 13) TLC developing chamber<br>(500-ml jar)   | 26) Reference agent, for example, hydralazine hydrochloride as 25 mg tablet or, alternatively, hydralazine hydrochloride as neat substance from commercial sources |

#### III. PREPARATION OF THE STOCK STANDARD SOLUTION

If reference tablets containing 25 mg of hydralazine hydrochloride are supplied, then wrap one tablet into aluminium foil and crush it down to a fine powder using a pestle. Carefully empty the aluminium foil over a 25-ml laboratory glass bottle and wash down all residual solids with 5 ml of water using a straight pipette. Close the bottle and shake for about three minutes until most of the solids are dissolved. Allow the solution to sit for an additional five minutes until undissolved residues settle below the supernatant liquid. The solution obtained should contain 5 mg of total hydralazine hydrochloride per ml and be labelled as 'Hydralazine Stock Standard Solution'. Freshly prepare this solution for each test. Continue to work with the clear or hazy supernatant liquid.

If the reference tablets are replaced by hydralazine hydrochloride powder of high purity close to 100%, then weigh in correctly about 0.3 g using the electronic pocket balance



supplied. Further, dissolve the powder in 60 ml of water thus obtaining again a solution containing 5 mg of total hydralazine hydrochloride per ml of water. Adjust the amount of water for dissolution when the weighing result differs from the target weight. In order to overcome the balance's in-built dynamic inertia, lift the weighing boat or tap the weighing pan with a pen or spatula each time after a few more milligrams have been added or removed. In order to ensure complete dissolution, observe the shaking and sitting times as mentioned above. Label as above. The final solution obtained should be clear without any observable residual solids. Freshly prepare this solution for each test.

#### IV. PREPARATION OF THE WORKING STANDARD SOLUTION 100% (UPPER WORKING LIMIT)

Pipette 1 ml of the stock standard solution into a 10-ml vial and add 3 ml of methanol. Close and shake the vial. The solution obtained should contain 1.25 mg of hydralazine hydrochloride per ml and be labelled as '*Hydralazine Working Standard Solution 100%*'.

This higher working standard solution represents a medicinal product of good quality containing 100% of hydralazine hydrochloride.

#### V. PREPARATION OF THE WORKING STANDARD SOLUTION 80% (LOWER WORKING LIMIT)

Pipette 1 ml of the stock standard solution into a 10-ml vial and add 4 ml of methanol using suitable graduated pipettes. Close and shake the vial. The solution obtained should contain 1 mg of total hydralazine hydrochloride per ml and be labelled as '*Hydralazine Working Standard Solution 80%*'.

This lower working standard solution represents a medicinal product of poor quality containing just 80% of the amount of hydralazine hydrochloride as stated on the product's label. In the current investigation, this level of hydralazine hydrochloride represents the lower acceptable limit for a given product. Pharmacopoeial limits do not apply in our context.

#### VI. PREPARATION OF THE STOCK SAMPLE SOLUTION FROM A LIQUID PRODUCT CLAIMING TO CONTAIN 20 MG OF HYDRALAZINE HCL PER AMPOULE OR VIAL

Take a sealed 1-ml ampoule or vial of an appropriate drug sampled in the field. Open the vial or ampoule and transfer the contents to a 10-ml laboratory glass bottle. Wash out the empty ampoule or vial with 3 ml of water and combine each wash solution with the sample solution.

##### LYOPHILIZED PRODUCT CLAIMING TO CONTAIN 20 MG OF HYDRALAZINE HCL PER AMPOULE OR VIAL

Take an ampoule or vial of a suitable medicinal product collected in the field. Open it and transfer the contents into a 25-ml laboratory glass bottle used as a sample container. Snap off the top of glass ampoules, wrap the ampoule body in aluminum foil and break it with a pestle before transferring all the glass fragments and the lyophilisate completely into the 25-ml laboratory bottle. Then extract everything with 4 ml of water. During the extraction, the label with its adhesive may also gradually detach, so that the entire solution may become turbid. However, this has no influence on the extraction of the hydralazine.

##### TABLET CLAIMING TO CONTAIN 25 MG OF HYDRALAZINE HCL PER UNIT

Take one whole sample tablet or capsule and extract the powder obtained with 5 ml of water using a straight pipette and a 10-ml laboratory glass bottle as sample container.

##### TABLET CLAIMING TO CONTAIN 50 MG OF HYDRALAZINE HCL PER UNIT

Take one whole sample tablet or capsule and extract the powder obtained with 10 ml of water using a straight pipette and a 25-ml laboratory glass bottle as sample container.

##### TABLET CLAIMING TO CONTAIN 100 MG OF HYDRALAZINE HCL PER UNIT

Take one whole sample tablet or capsule and extract the powder obtained with 20 ml of water using a straight pipette and a 25-ml laboratory glass bottle as sample container.

All stock sample solutions produced should finally contain 5 mg of total hydralazine hydrochloride per ml and be labelled as '*Hydralazine Stock Sample Solution*'. Freshly prepare these solutions for each test. Continue working with the hazy liquid obtained.

## VII. PREPARATION OF THE WORKING SAMPLE SOLUTION

Pipette 1 ml of the stock sample solution into a 10-ml vial and add 3 ml of methanol. Close, shake and label the vial as '*Hydralazine Working Sample Solution*'.

The expected concentration of hydralazine hydrochloride in the working sample solution is 1.25 mg per ml and it should match the concentration of hydralazine hydrochloride of the higher working standard solution produced above.

## VIII. SPOTTING

Mark an origin line parallel to and about 1.5 cm from the bottom edge of the chromatoplate and apply 2 µl of each test and standard solution as shown in the picture opposite using the microcapillary pipettes supplied.

Up to five spots can be placed on a plate. Check the uniformity of all spots using UV light of 254 nm. All spots should be circular in shape and equally spaced across the origin line. Although their intensities might differ, their diameters never should. Different intensities are due to residual amounts of excipients or different drug concentrations in the sample solutions. A difference in spot size, however, relates to poor spotting. Repeat this step if homogeneous spotting is not achieved first time.

Gently dry the spots. To do this, hold the chromatoplate with the supplied tweezers in the hot air stream directly above the heating plate for about thirty seconds. Shake the TLC plate constantly and each time the chromatoplate moves downwards, its underside is allowed to touch the surface of the heating plate for fractions of a second.

## IX. DEVELOPMENT

Using suitable graduated pipettes, add 10 ml of methanol, 5 ml of acetone and 5 ml of ammonia solution 25% to the jar serving as TLC developing chamber. Close the chamber and mix thoroughly. Line the chamber's wall with filter paper and wait for about 15 minutes thus ensuring saturation of the chamber with solvent vapour. Carefully place the loaded TLC plate into the jar. Close the jar and develop the chromatoplate until the solvent front has moved about three-quarters of the length of the plate, the developing time being about 20 minutes. Remove the TLC plate from the chamber, mark the solvent front and allow excess solvent to air dry for about five minutes.

## X. DETECTION

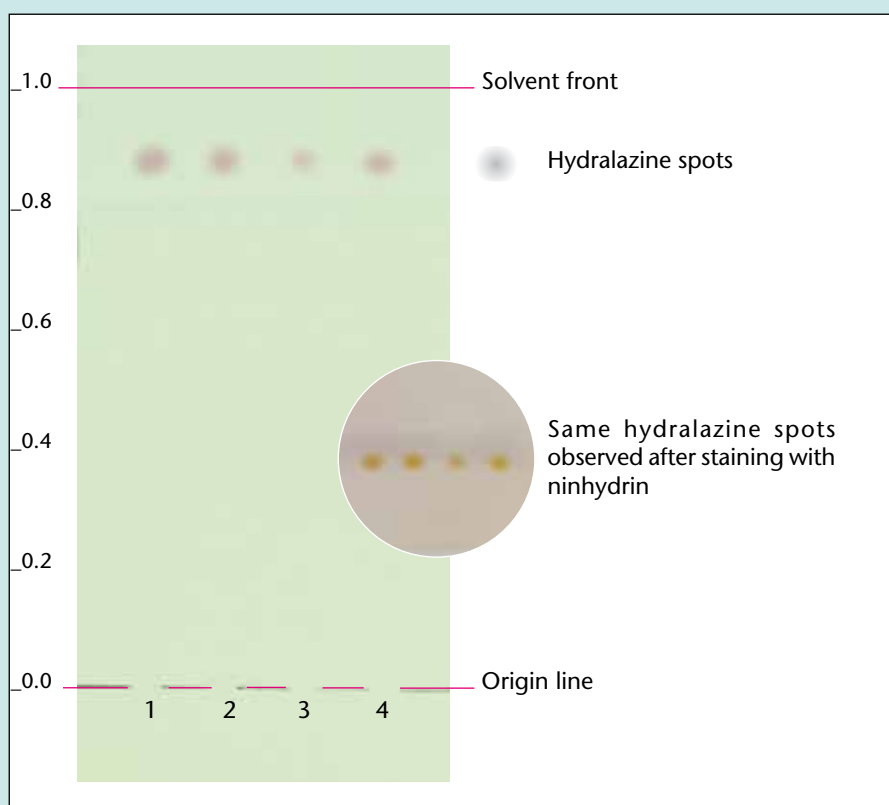
After drying off all solvent residues, view the chromatography plate under UV light at 254 nm with the battery driven lamp provided. Use this method of detection for both, identification and quantification of hydralazine.

For further hydralazine detection, perform staining with ninhydrin. For this, dissolve about 3 g of ninhydrin (about 10 times a well-filled spatula) in a mixture of 150 ml of methanol and 30 ml of acetic acid solution 96%. Use the plastic beaker supplied to accommodate the staining solution. This will allow dipping the chromatoplate into the solution using a pair of tweezers. Instantly remove the plate from the beaker, let surplus solution run down onto paper tissue and finally dry the back of the plate using paper tissue again. Continue to dry off all staining solution on a hot plate and observe how the spots for hydralazine are gradually becoming visible.

The ninhydrin staining process is illustrated in full on page 36 of the main manual. Note that skin contaminated with ninhydrin solution will be stained as well. However, this is not dangerous to health and the violet spots will disappear after about a day or two.

CHROMATOPLATE OBSERVED UNDER  
UV LIGHT OF 254 NM

- Run No.1:  
Upper working standard representing  
100% of total hydralazine
- Run No.2:  
A product of good quality with acceptable  
hydralazine content
- Run No.3:  
A product of poor quality with unaccept-  
able low hydralazine content
- Run No.4:  
Lower working standard representing  
80% of total hydralazine



XI. OBSERVATIONS MADE AT 254 NM

A dark spot at a travel distance of about 0.88 indicates the presence of hydralazine in the test solution. Additional strong spots generated by the test solution would point at other drugs or hydralazine degradation, the latter case being more likely when associated with a smaller principal spot. A smaller principal spot from the test solution may also indicate a poor hydralazine content and no spot at all a complete absence of hydralazine. Auxiliary agents incorporated in different finished products might cause some fainter spots either travelling up to the solvent front or emerging near or on the origin line.

XII. OBSERVATIONS MADE AT DAY-  
LIGHT AFTER NINHYDRIN  
STAINING

All hydralazine spots that were previously made visible with UV light at 254 nm now turn reddish-brown and later fade to orange-brown. If the chromatoplate still contains residues of ammonia solution from the mobile phase, the entire plate could be coloured at the end of the day. Therefore, semi-quantifications are not always conclusive, but ninhydrin staining is good for further hydralazine identification.

XIII. RESULTS & ACTIONS TO BE TAKEN

The hydralazine spot in the chromatogram obtained with the test solution must correspond in terms of colour, size, intensity, shape and travel distance to that in the chromatogram obtained with the lower and higher standard solution. This result must be obtained for each method of detection. If this is not achieved, repeat the run from scratch with a second sample. Reject the batch if the drug content cannot be verified in a third run. For a second opinion, refer additional samples to a fully-fledged drug quality control laboratory. Retain samples and put the batch on quarantine until a final decision on rejection or release has been taken. For documentation purposes, take pictures of all the readings with a digital camera turning off the flash first.

## 7.112 Rivaroxaban

### Primary Screening on Product Deficiencies by Physical Testing

#### I. PHYSICAL TESTING

During visual inspection, search for deficiencies on labelling, packaging and dosage forms as described in the opening chapters on general methods and operations of the main manual and report the findings. Consider to take pictures, for example, with a smartphone camera. Each tablet usually contains 2.5, 10, 15 or 20 mg of rivaroxaban. Verify the total weight of tablets and capsules using the electronic pocket balance provided.

All quick release rivaroxaban tablet and capsule formulations have to pass the disintegration test as described at the beginning of the main manual. They should disintegrate in water at 37 °C in less than 30 minutes. It is a major defect if an instant release formulation does not pass this test.

#### II. RESULTS & ACTIONS TO BE TAKEN

Medicinal products from unusually cheap sources, medicinal products with missing or incorrect accompanying documents and medicinal products with defective dosage forms, packaging or with incomplete, damaged or missing labels or with labels in a foreign language or medicinal products held under poor storage conditions should be subjected to a thin-layer chromatographic test.

### Verification of Drug Identity and Content by Thin-Layer Chromatography

#### I. PRINCIPLE

Rivaroxaban is extracted from tablets or capsules with a known volume of concentrated acetic acid solution and is subsequently checked for identity and content by thin-layer chromatography (TLC) in comparison with a suitable secondary standard.

#### II. EQUIPMENT AND REAGENTS

- 1) Pestle
- 2) Aluminium foil
- 3) Funnel
- 4) Spatula
- 5) Label tape
- 6) Marker pen
- 7) Pencil and ruler
- 8) 10-ml vials
- 9) Set of graduated pipettes (1 to 25 ml)
- 10) Set of laboratory glass bottles (25 to 100 ml)
- 11) Merck TLC aluminium plates pre-coated with silica gel 60 F<sub>254</sub>, size 5x10 cm
- 12) Glass microcapillaries (2-µl filling capacity)
- 13) TLC developing chamber (500-ml jar)
- 14) Hot plate
- 15) Filter paper
- 16) Pair of scissors
- 17) Pair of tweezers
- 18) UV light of 254 nm
- 19) Methanol
- 20) Ethyl acetate
- 21) Acetic acid solution 96%
- 22) Reference agent, for example, rivaroxaban 10 mg tablets

### III. PREPARATION OF THE STOCK STANDARD SOLUTION

The preparation of the stock standard solution requires an authentic medicinal product for reference purposes, for example, tablets containing 10 mg of rivaroxaban. Wrap up one reference tablet into aluminium foil and crush it down to a fine powder using a pestle. Carefully empty the aluminium foil over a 25-ml laboratory glass bottle and wash down all residual solids with 10 ml of acetic acid solution 96% using a graduated pipette. Close the lab bottle and shake it for about three minutes. Allow the solution to stand for an additional five minutes until undissolved residues settle below the supernatant liquid. The hazy solution obtained should contain 1 mg of total rivaroxaban per ml and be labelled as '*Rivaroxaban Stock Standard Solution*'. Freshly prepare this solution for each test. Continue working with the hazy liquid obtained.

### IV. PREPARATION OF THE WORKING STANDARD SOLUTION 100% (UPPER WORKING LIMIT)

Pipette 1 ml of the stock standard solution into a 10-ml vial and add 1 ml of acetic acid solution 96% using suitable graduated pipettes. Close and shake the vial. The solution obtained should contain 0.5 mg of total drug per ml and be labelled as '*Rivaroxaban Working Standard Solution 100%*'.

This higher working standard solution represents a medicinal product of good quality containing 100% of rivaroxaban.

### V. PREPARATION OF THE WORKING STANDARD SOLUTION 80% (LOWER WORKING LIMIT)

Pipette 1 ml of the stock standard solution into a 10-ml vial and add 1.5 ml of acetic acid solution 96% using suitable graduated pipettes. Close and shake the vial. The solution obtained should contain 0.4 mg of total rivaroxaban per ml and be labelled as '*Rivaroxaban Working Standard Solution 80%*'.

This lower working standard solution represents a medicinal product of poor quality containing just 80% of the amount of rivaroxaban as stated on the product's label. In the current investigation, this level of rivaroxaban represents the lower acceptable limit for a given product. Pharmacopoeial limits do not apply in our context.

### VI. PREPARATION OF THE STOCK SAMPLE SOLUTION FROM A PRODUCT CLAIMING TO CONTAIN 2.5 MG OF RIVAROXABAN PER UNIT

Take a whole tablet or capsule from a suitable medicinal product sampled in the field. As usual, tablets are wrapped in aluminium foil and crushed into a fine powder. Transfer all the powder obtained into a 10-ml laboratory glass bottle. Powder obtained from sample capsules should be placed directly in the bottle adding the empty cap and body shells last. For extraction, add 2.5 ml of acetic acid solution 96% using a suitable graduated pipette. Close the bottle and shake it for about 3 minutes. Allow the solution to stand for another 5 minutes and ensure that any undissolved residue settles below the supernatant liquid.

#### 10 MG OF RIVAROXABAN PER UNIT

Place the powder obtained from a whole sample tablet or capsule into a 25-ml laboratory glass bottle, add 10 ml of acetic acid solution 96% with a suitable graduated pipette and extract the rivaroxaban. Continue working as described above.

#### 15 MG OF RIVAROXABAN PER UNIT

Place the powder obtained from a whole sample tablet or capsule into a 25-ml laboratory glass bottle, add 15 ml of acetic acid solution 96% with a suitable graduated pipette and extract the rivaroxaban. Continue working as described above.

#### 20 MG OF RIVAROXABAN PER UNIT

Place the powder obtained from a whole sample tablet or capsule into a 25-ml laboratory glass bottle, add 20 ml of acetic acid solution 96% with a suitable graduated pipette and extract the rivaroxaban. Continue working as described above.

All stock sample solutions produced should finally contain 1 mg of total rivaroxaban per ml and be labelled as '*Rivaroxaban Stock Sample Solution*'. Freshly prepare these solutions for each test. Continue working with the clear or hazy liquid obtained.

## VII. PREPARATION OF THE WORKING SAMPLE SOLUTION

Pipette 1 ml of the stock sample solution into a 10-ml vial and add 1 ml of acetic acid solution 96% using suitable graduated pipettes. Close and shake the vial and label as '*Rivaroxaban Working Sample Solution*'.

The expected concentration of rivaroxaban in this working sample solution is 0.5 mg per ml and it should correspond to the concentration of rivaroxaban of the higher working standard solution produced above.

## VIII. SPOTTING

Mark an origin line parallel to and about 1.5 cm from the bottom edge of the chromatoplate and apply 2 µl of each test and standard solution as shown in the picture opposite using the microcapillary pipettes supplied.

Up to five spots can be placed on a plate. Check the uniformity of all spots using UV light of 254 nm. All spots should be circular in shape and equally spaced across the origin line. Although their intensities might differ, their diameters never should. Different intensities are due to residual amounts of excipients or different drug concentrations in the sample solutions. A difference in spot size, however, relates to poor spotting. Repeat this step if homogeneous spotting is not achieved first time.

Gently dry the spots. To do this, hold the chromatoplate with the supplied tweezers in the hot air stream directly above the heating plate for about one minute. Shake the TLC plate constantly and each time the chromatoplate moves downwards, its underside is allowed to touch the surface of the heating plate for fractions of a second.

## IX. DEVELOPMENT

Using suitable graduated pipettes, add 15 ml of ethyl acetate, 5 ml of methanol and 0.5 ml of acetic acid solution 96% to the jar serving as TLC developing chamber. Close the chamber and mix thoroughly. Line the chamber's wall with filter paper and wait for about 15 minutes thus ensuring saturation of the chamber with solvent vapour. Carefully place the loaded TLC plate into the jar. Close the jar and develop the chromatoplate until the solvent front has moved about three-quarters of the length of the plate, the developing time being about 15 minutes. Remove the TLC plate from the chamber, mark the solvent front and allow excess solvent to evaporate by gentle drying. To do this, hold the chromatoplate with the supplied tweezers in the hot air stream directly above the heating plate for about two minutes. Shake the TLC plate constantly and each time the chromatoplate moves downwards, its underside is allowed to touch the surface of the heating plate for fractions of a second.

## X. DETECTION

After drying off all solvent residues, view the chromatography plate under UV light at 254 nm with the battery driven lamp provided. Use this method of detection of both, identification and quantification of rivaroxaban.

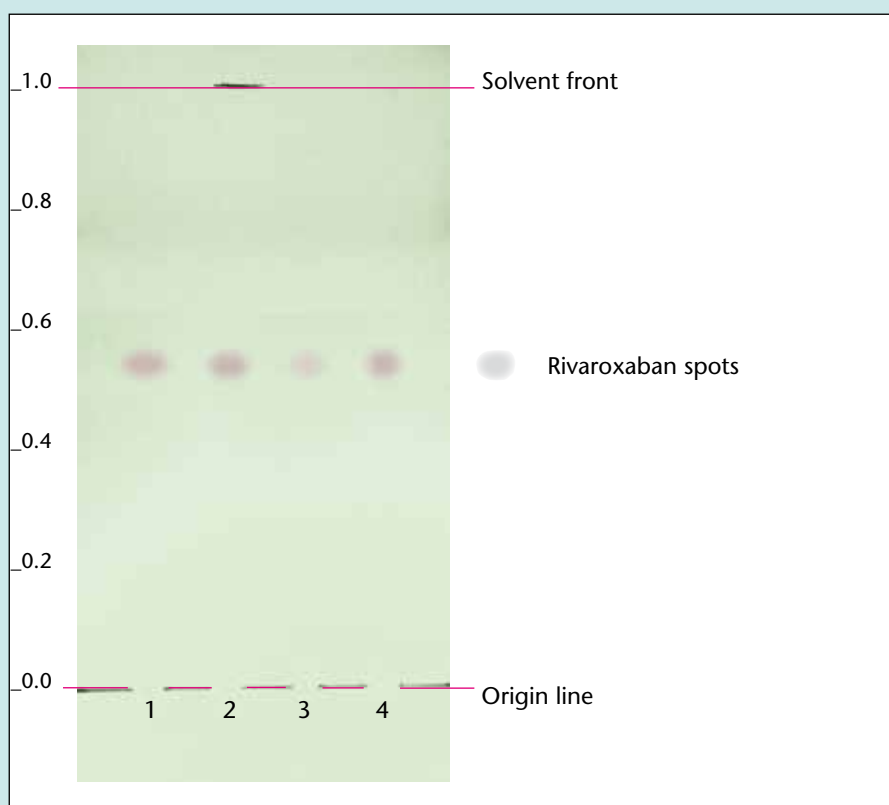
CHROMATOPLATE OBSERVED UNDER  
UV LIGHT OF 254 NM

Run No.1:  
Upper working standard representing  
100% of total rivaroxaban

Run No.2:  
A product of good quality with acceptable  
rivaroxaban content

Run No.3:  
A product of poor quality with unaccept-  
able low rivaroxaban content

Run No.4:  
Lower working standard representing  
80% of total rivaroxaban



XI. OBSERVATIONS MADE AT 254 NM

A dark spot at a travel distance of about 0.53 indicates the presence of rivaroxaban in the test solution. Additional strong spots produced by the test solution would point at other drugs or rivaroxaban degradation, the latter case being more likely when associated with a smaller principal spot. A smaller principal spot from the test solution may also indicate a poor rivaroxaban content and no spot at all a complete absence of rivaroxaban. Related active agents, for example apixaban, would appear to have a relative retention factor of about 0.45 and even produce white fluorescence when viewed under UV light at 366 nm emitted by a powerful laboratory lamp. Excipients contained in various finished products can cause some fainter spots that either migrate to the solvent front or appear near or on the line of origin. Lactose remains invisible, but additional staining tests with sulphuric acid and heat indicate that it would show up at about 0.10 if present in the formulation.

XII. RESULTS & ACTIONS TO BE TAKEN

The rivaroxaban spot in the chromatogram obtained with the test solution must correspond in terms of colour, size, intensity, shape and travel distance to that in the chromatogram obtained with the lower and higher standard solution. This result must be obtained for each method of detection. If this is not achieved, repeat the run from scratch with a second sample. Reject the batch if the drug content cannot be verified in a third run. For a second opinion, refer additional samples to a fully-fledged drug quality control laboratory. Retain samples and put the batch on quarantine until a final decision on rejection or release has been taken. For documentation purposes, take pictures of all the readings with a digital camera turning off the flash first.

## Primary Screening on Product Deficiencies by Physical Testing

### I. PHYSICAL TESTING

During visual inspection, search for deficiencies on labelling, packaging and dosage forms as described in the opening chapters on general methods and operations of the main manual and report the findings. Consider to take pictures, for example, with a smartphone camera. Each tablet usually contains 1, 2, 3 or 5 mg of warfarin sodium salt. Other dosage strengths are known to exist. Verify the total weight of tablets and capsules

using the electronic pocket balance provided. All quick release warfarin tablet and capsule formulations have to pass the disintegration test as described at the beginning of the main manual. They should disintegrate in water at 37 °C in less than 30 minutes. It is a major defect if an instant release formulation does not pass this test.

### II. RESULTS & ACTIONS TO BE TAKEN

Medicinal products from unusually cheap sources, medicinal products with missing or incorrect accompanying documents and medicinal products with defective dosage forms, packaging or with incomplete, damaged or missing labels or with labels in a foreign language or medicinal products held under poor storage conditions should be subjected to a thin-layer chromatographic test.

## Verification of Drug Identity and Content by Thin-Layer Chromatography

### I. PRINCIPLE

Warfarin sodium, with or without isopropanol content, is extracted from tablets or capsules with a known volume of methanol and is subsequently checked for identity and content by thin-layer chromatography (TLC) in comparison with a suitable secondary standard.

### II. EQUIPMENT AND REAGENTS

- 1) Pestle
- 2) Aluminium foil
- 3) Funnel
- 4) Spatula
- 5) Label tape
- 6) Marker pen
- 7) Pencil and ruler
- 8) 10-ml vials
- 9) Set of graduated pipettes (1 to 25 ml)
- 10) Set of laboratory glass bottles (25 to 100 ml)
- 11) Merck TLC aluminium plates pre-coated with silica gel 60 F<sub>254</sub> size 5x10 cm
- 12) Glass microcapillaries (2-µl filling capacity)
- 13) TLC developing chamber (500-ml jar)
- 14) Hot plate
- 15) Filter paper
- 16) Pair of scissors
- 17) Pair of tweezers
- 18) UV light of 254 nm
- 19) Iodine chamber
- 20) Toluene
- 21) Methanol
- 22) Ethyl acetate
- 23) Acetic acid solution 96%
- 24) Reference agent, for example, warfarin sodium 5 mg tablets



### III. PREPARATION OF THE STOCK STANDARD SOLUTION

The preparation of the stock standard solution requires an authentic medicinal product for reference purposes, for example, tablets containing 5 mg of warfarin sodium salt. Wrap up one reference tablet into aluminium foil and crush it down to a fine powder using a pestle. Carefully empty the aluminium foil over a 10-ml laboratory glass bottle and wash down all residual solids with 5 ml of methanol using a graduated pipette. Close the lab bottle and shake for about three minutes until most of the solids are dissolved. Allow the solution to stand for an additional five minutes until undissolved residues settle below the supernatant liquid. The solution obtained should contain 1 mg of total warfarin sodium per ml and be labelled as '*Warfarin Stock Standard Solution*'. Freshly prepare this solution for each test. Continue to work with the clear or hazy supernatant liquid.

### IV. PREPARATION OF THE WORKING STANDARD SOLUTION 100% (UPPER WORKING LIMIT)

The stock standard solution requires no further dilution. It already represents the final working concentration of 1 mg of total warfarin sodium per ml. For more convenient handling, some of the supernatant liquid may be transferred to a 10-ml vial.

This higher working standard solution represents a medicinal product of good quality containing 100% of warfarin sodium.

### V. PREPARATION OF THE WORKING STANDARD SOLUTION 80% (LOWER WORKING LIMIT)

Pipette 2 ml of the stock standard solution into a 10-ml vial and add 0.5 ml of methanol using suitable graduated pipettes. Close and shake the vial. The solution obtained should contain 0.8 mg of total warfarin sodium per ml and be labelled as '*Warfarin Working Standard Solution 80%*'.

This lower working standard solution represents a medicinal product of poor quality containing just 80% of the amount of warfarin sodium as stated on the product's label. In the current investigation, this level of warfarin sodium represents the lower acceptable limit for a given product. Pharmacopoeial limits do not apply in our context.

### VI. PREPARATION OF THE STOCK SAMPLE SOLUTION FROM A PRODUCT CLAIMING TO CONTAIN 2 MG OF WARFARIN SODIUM PER UNIT

Take three whole tablets or capsules from a suitable medicinal product sampled in the field. As usual, tablets are wrapped up into aluminium foil and crushed down to a fine powder. Transfer all the powder obtained into a 25-ml laboratory glass bottle. Powder obtained from sample capsules should be placed directly in the bottle adding the empty cap and body shells last. For extraction, add 6 ml of methanol using a suitable graduated pipette. Then, close the bottle and shake for about three minutes until most of the solids are dissolved. Allow the solution to stand for an additional five minutes until undissolved residues settle below the supernatant liquid.

#### 3 MG OF WARFARIN SODIUM PER UNIT

Place the powder obtained from two whole sample tablets or capsules into a 25-ml laboratory glass bottle, add 6 ml of methanol with a suitable graduated pipette and extract the warfarin sodium. Continue working as described above.

#### 5 MG OF WARFARIN SODIUM PER UNIT

Place the powder obtained from one whole sample tablet or capsule into a 10-ml laboratory glass bottle, add 5 ml of methanol with a suitable graduated pipette and extract the warfarin sodium. Continue working as described above.

All stock sample solutions produced should finally contain 1 mg of total warfarin sodium per ml and be labelled as '*Warfarin Stock Sample Solution*'. Freshly prepare these solutions for each test. Continue working with the clear or hazy supernatant liquids.

## VII. PREPARATION OF THE WORKING SAMPLE SOLUTION

The stock sample solutions require no further dilution. They already represent the final working concentration of 1 mg of warfarin sodium per ml. If prepared from a high quality product, the sample solutions should match the concentration of warfarin sodium of the higher working standard solution produced above. For more convenient handling, some of the supernatant liquid may be transferred to a 10-ml vial.

## VIII. SPOTTING

Mark an origin line parallel to and about 1.5 cm from the bottom edge of the chromatoplate and apply 2 µl of each test and standard solution as shown in the picture opposite using the microcapillary pipettes supplied.

Up to five spots can be placed on a plate. Check the uniformity of all spots using UV light of 254 nm. All spots should be circular in shape and equally spaced across the origin line. Although their intensities might differ, their diameters never should. Different intensities are due to residual amounts of excipients or different drug concentrations in the sample solutions. A difference in spot size, however, relates to poor spotting. Repeat this step if homogeneous spotting is not achieved first time.

Allow the spots to air-dry for one to two minutes until the methanol has almost completely disappeared. Avoid any heat drying.

## IX. DEVELOPMENT

Using suitable graduated pipettes, add 15 ml of toluene, 5 ml of ethyl acetate and 1 ml of acetic acid solution 96% to the jar serving as TLC developing chamber. Close the chamber and mix thoroughly. Line the chamber's wall with filter paper and wait for about 15 minutes thus ensuring saturation of the chamber with solvent vapour. Carefully place the loaded TLC plate into the jar. Close the jar and develop the chromatoplate until the solvent front has moved about three-quarters of the length of the plate, the developing time being about 15 minutes. Remove the TLC plate from the chamber, mark the solvent front and allow excess solvent to evaporate by gentle drying. To do this, hold the chromatoplate with the supplied tweezers in the hot air stream directly above the heating plate for about two minutes. Shake the TLC plate constantly and each time the chromatoplate moves downwards, its underside is allowed to touch the surface of the heating plate for fractions of a second.

## X. DETECTION

After drying off all solvent residues, view the chromatographic plate under UV light at 254 nm with the battery driven lamp provided. For further identification and quantification of warfarin, stain the chromatoplate with iodine vapours. View the stained chromatography plate again under UV light at 254 nm. Some previously observed spots might appear stronger allowing better readings. All of the above detection methods also apply to phenprocoumon, an active pharmaceutical ingredient closely related to warfarin.

## XI. OBSERVATIONS MADE AT 254 NM

A dark spot at a travel distance of about 0.38 indicates the presence of warfarin in the test solution. Additional strong spots generated by the test solution would point at other drugs or warfarin degradation, the latter case being more likely when associated with a smaller principal spot. A smaller principal spot from the test solution may also

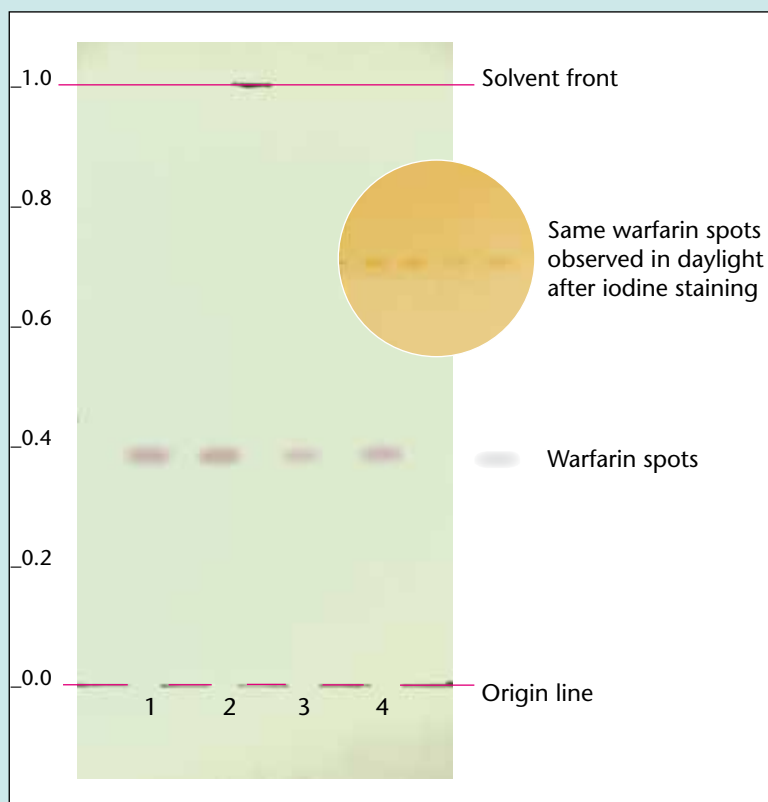
## CHROMATOPLATE OBSERVED UNDER UV LIGHT OF 254 NM

Run No.1:  
Upper working standard representing  
100% of total warfarin

Run No.2:  
A product of good quality with acceptable  
warfarin content

Run No.3:  
A product of poor quality with unaccept-  
able low warfarin content

Run No.4:  
Lower working standard representing  
80% of total warfarin



indicate a poor warfarin content and no spot at all a complete absence of warfarin. The closely related phenprocoumon would show a relative retention factor of about 0.52. Auxiliary agents incorporated in different finished products might cause some fainter spots either travelling up to the solvent front or emerging near or on the origin line.

## XII. OBSERVATIONS MADE IN DAY- LIGHT AFTER IODINE STAINING

When the TLC plate is further exposed to iodine vapour, all warfarin spots previously observed at 254 nm now turn light brown, with different shades and sizes indicating different warfarin concentrations. Phenprocoumon behaves very similarly here. However, the staining is relatively weak for both compounds.

## XIII. RESULTS & ACTIONS TO BE TAKEN

The warfarin spot in the chromatogram obtained with the test solution must correspond in terms of colour, size, intensity, shape and travel distance to that in the chromatogram obtained with the lower and higher standard solution. This result must be obtained for each method of detection. If this is not achieved, repeat the run from scratch with a second sample. Reject the batch if the drug content cannot be verified in a third run. For a second opinion, refer additional samples to a fully-fledged drug quality control laboratory. Retain samples and put the batch on quarantine until a final decision on rejection or release has been taken. For documentation purposes, take pictures of all the readings with a digital camera turning off the flash first.

- Detecting falsified and substandard medicines in low and middle-income countries
- Protecting consumers and medicines supply chains
- Boosting medicines testing capacities for priority medicines
- Assisting in post-marketing medicines quality monitoring
- Complementing the work of existing medicines control laboratories

The GPHF-Minilab™ is a unique miniature laboratory which comes with affordable test methods for a rapid and easy detection of falsified and substandard medicines as entry-level technology for resource limited health settings in low- and middle-income countries.

In more than twenty years of project work, the GPHF-Minilab™ has proven its suitability in more than a 100 countries.

This supplement to the Minilab Manual expands the list of cardiovascular medicines to overall twenty active pharmaceutical ingredients including their fixed-dose combinations to treat cardiovascular disorders.

The method inventory of the GPHF-Minilab™ manual now includes a collection of test methods for 113 active pharmaceutical ingredients for rapid quality verification of a wide range of finished pharmaceutical products.



Global Pharma Health Fund  
[www.gphf.org](http://www.gphf.org)