HPLC Assay And Stability Testing of Adrenaline from Compounded Rectal Suppositories

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The adrenaline content of Tonogen (adrenaline)+benzocaine HCl+Peru balsam+oleum ricini+adeps solidus rectal suppository, a commonly prescribed non-official topical compounded preparation for piles (hemorrhoids) in Hungary, manufactured in a community pharmacy by fusion molding, was assayed and its stability during storage at 5-6 °C for 28 days in primary and secondary packaging was determined by a validated HPLC method. The 68 rectal suppositories manufactured showed a variance of 114% to 58% in their actual adrenaline contents compared to the nominal average adrenaline concentration in the bulk mass at time t0 (the latter value was measured in the last suppository). Within the group of suppositories included in the stability study, the lowest measured concentration (suppository Nr. 41; 7.64 μg/g) was 77% of the nominal average bulk concentration at time t1, while the highest measured concentration (suppository Nr. 46; 11.35 μg/g) was 114% of the nominal average bulk concentration at time t0. During the storage period, the adrenaline content of the suppositories did not show any significant decrease. The inhomogeneity of adrenaline contents found among suppositories did not show any significant trend in the function of manufacturing order (if the last suppositories manufactured were excluded).

1. Introduction

The use of adrenaline-containing combination rectal suppositories for the symptomatic treatment of piles (hemorrhoids) has been reported among domestic compounded (magistral) formulations in the literature since the early 1910s [1, 2, 3].

In the 1943 edition of the Official Register of Medicinal Products, there were already 10 registered, factory-manufactured preparations presented with full qualitative and quantitative composition: **Anorrhal** suppository /Austria-Medichemia/, **Anugen** suppository /Son/, **Nodex** suppository / Pharmacia/, **Nodil** suppository /Wander/, **Nohäsa** suppository /Chinoin/, **Rectal** suppository /Bayer A/, **Sanolan** suppository /Christian/, **Sandrect** suppository /Spolio/, **Mydi** suppository /Mydi-Medichemia/ and **Tonogen** suppository /Richter/ [4].

During the socialist period, the **Nohäsa/Nohaesa** suppository (and ointment) /Chinoin/ [5] remained the only one authorised product from the above group, which was marketed from 1953 as **Hemorid** rectal suppository with adrenaline component [6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17] until 2009. On 28. 05. 2009, a variation of marketing authorization was approved by the National Institute of Pharmacy [Pharmamagist Ltd. personal information], when adrenaline was removed from the composition and ephedrine HCl alone was left as vasoconstrictor.

The above mentioned compositions are typically based on cocoa butter (butyrum cacao) vehicle,

- *in all cases with adrenaline vasoconstrictor (the Nohaesa/Hemorid suppository had ephedrine HCl, too),*
- *in all cases with some form of local anaesthetic (e.g. cocaine, novocaine, procaine, benzocaine, menthol, trichloroisobutanol),*
- *in all cases with an antibacterial component (diiodoparaenol sulphonic acid sodium salt, oxyiodogallic acid or bismuth salt, bismuth subnitrate, para-aminobenzoic acid, resorcinol, mercury-free dermaphorin, pyoktaninum auraminum, aetheroleum millefolii, camphor, Peru balsam),*
- *occasionally with an active substance used as haemostatic agent (hydrastinin),*
- *occasionally with a facilitator of re-epithelization and/or anti-inflammatory component (zinc oxide,*

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Hamamelis virginia extract, Aesculus hippocastanum extract, Matricaria chamomilla extract) [4].

Suppositoria haemorrhoidalia cum cocaino with cocaine HCl, adrenaline solution, resorcinol, boric acid, Peru balsam, bismuth oxyiodide gallate and cocoa butter were included in the First (1939) [18] and Second (1948) [19] Editions of Formulae Normales (Hungarian National Formulary of compounded medicinal preparations).

However, in the Third Edition (1954) of the Formulae Normales [20], adrenaline and cocaine were omitted. The new composition, *Suppositurium haemorrhoidale analgeticum*, contained ephedrine HCl, tetracaine HCl, menthol, resorcinol, Peru balsam, boric acid, bismuth subgallate and cocoa butter.

From that on, all subsequent editions of the Formulae Normales maintained the exclusive use of ephedrine HCl in rectal suppositories indicated for the symptomatic treatment of piles. No explanation for this decision has been given in published sources, but until 28. 05. 2009, adrenaline continued to be included in the composition of the factory-made Hemorid rectal suppositories (see above).

In 1931, Elek Mezei, a pharmacy owner in Újpest, stated in the Hungarian pharmaceutical newspaper 'Gyógyszerészi Hírlap' as fact, but without presenting analytical data, that "*It is incorrect to prescribe adrenaline or Tonogen (a factory-made adrenaline solution) mixed with cocoa butter for rectal suppositories. Adrenaline and Tonogen are highly oxidizable. Adrenaline is exposed to so much oxygen from air and vehicle during mixing that it decomposes and becomes completely ineffective. Instead of this compound, ephedrine, which remains unchanged when mixed with cocoa butter, can be recommended as a substitute in rectal suppositories with exactly the same effect*' [21].

The compounded rectal suppository we have tested had the following composition:

Benzocaine:	10 g
Balsamum peruvianum:	4 g
Tonogen (1 mg/ml adrenaline):	1 ampoule
Adeps solidus:	79.2 g
Ricini oleum virginale:	q. s.

As presented above, the traditional method of preparation of medicated suppositories is by pressing (using cocoa butter). Nowadays, however, fusion moulding has become common, in this case using adeps solidus (hard fat).

Apart from this, however, the composition of this compounded rectal suppository practically follows the Hungarian tradition of the last century or more (vasoconstrictor + local analgesic + antibacterial, wound-healing components).

However, neither the international nor the national literature has reported any assay method for adrenaline from rectal suppositories and stability data.

Therefore, we aimed to develop a suitable assay method and investigate the stability of the adrenaline content in the above compounded rectal suppository, which is still widely used in Hungary, until the end of the 28-day storage period in refrigerator, as recommended in the everyday practice.

The Ph. Eur. monographs of adrenaline [22] and adrenaline tartrate [23], respectively, specify the same ion-pair HPLC separation for the test of related substances, using sodium octanesulfonate ion-pairing reagent in order to increase the retention of the highly polar compounds on common end-capped octadecylsilyl silica gel stationary phases. Ion-pairing reagents have also been used for the quantitative determination of adrenaline in drug products, more precisely in injection solutions [24, 25]. Other studies on adrenaline stability in injection solutions have shown that the retention of adrenaline does not necessarily require the use of ion-pair chromatography when the stationary phase is replaced by a special, 100% aqueous-compatible C18-phase [26] or biphenyl-phase [27]. Uses of further unique phases, like another aqueous-compatible C18- [28], phenyl-ethyl-, phenyl-hexyl- [29] or pentafluorophenylpropyl-phases [30], have also been described in application notes for the separation of catecholamine compounds.

Unfortunately, these application notes have not revealed information about their performance when any matrix would be present. The assays of real drugs, that we found in the literature, have been applied to injection solutions only, representing simple matrix compositions like sterile water or 5% dextrose in water solution (D5W), occasionally supplemented by some salts and preservatives [24, 25, 26, 27, 31]. Peru balsam suppositories represent a much more complex sample type that have huge amounts of matrix components of various polarity: ca. 10% benzocaine (polar), ca. 80% adeps solidus (non-polar) and ca. 5-5% Peru balsam and Ricini oleum virginale (complex mixtures of natural compounds). Moreover, the working ranges of the reviewed adrena-

line assays in injection solutions have varied between 0.011 mg/ml, at 13 orders of magnitude higher than anticipated for prepared sample solutions of Peru balsam suppositories. Considering all these challenging circumstances, we also aimed to validate the resulting assay method before using it for the stability study.

2. Materials and methods

The development and validation of the HPLC-UV method for the assay of adrenaline and the stability testing were carried out at the Budapest site of QualcoDuna Proficiency Testing Hungary Nonprofit Ltd. between 10. 01. 2024 and 05. 06. 2024.

2.1 Development of the assay method

Samples, chemicals and equipment:

Samples for the method development:

The bulk suppository mass was prepared in a community pharmacy laboratory using the following actual weighings:

1. *Pulverization of benzocaine: weighed in into a mortar:* *10.0 g;*
   after pulverization and transfer to a mixing bowl: *9.9 g*
2. *Weighing in of adeps solidus:* *79.2 g*
3. *Weighing in of Peru balsam (Balsamum peruvianum):* *4.0 g*
4. *Weighing in of ricini oleum virginale:* *3.2 g*
5. *Weighing in of Tonogen injection:* *1.1 g*

Total weight of the bulk suppository mass: 97.4 g

Nominal average adrenaline concentration in the bulk suppository mass: **10.3 μg/g**

30 pcs rectal suppositories were moulded from the bulk mass with a nominal mass of 2.7 g each. The leftover of the bulk suppository mass was discarded.

30 pcs 'Placebo' suppositories were prepared in the same way but without the Tonogen solution.

Reference materials:

Tonogen ampoule, containing 1 mg/ml adrenaline; Batch number: A24092A; Shelf life: 04. 2026.

Reagents: Potassium dihydrogen phosphate: Supelco, EMSURE for analysis

Solvents, solutions:

Acetonitrile: PanReac AppliChem UHPLC supergradient

Water for HPLC: StakPure
OmniPure UV 0.055 μS/cm

Phosphate buffer: 50 mM KH_2PO_4 solution, pH=3.0

Equipment:

Ultrasonic bath: Elma, Elmasonic S120

Centrifuges: Hermle Z366; Eppendorf 5430 R

Centrifuge tubes: 50 ml volume, falcon;
Eppendorf 2 ml,

Automatic pipette:
Eppendorf 10-100 μl, 100-1000 μl

Laboratory scale: AA Labor MT-EXC50002

HPLC with autosampler (non-thermostatted), column thermostat and UV detector (DAD): Agilent 1100 series

Preparation of the samples for HPLC analysis:

One suppository was placed in a 50 ml centrifuge tube and weighed. Then 20 ml of 50 mM KH_2PO_4 pH=3.0 buffer heated to 50 °C was added. The tube was shaken and the content sonicated in an ultrasonic bath in hot water for 15 min, shaking the tube manually every 5 min. After extraction, 50 mM KH_2PO_4 pH=3.0 buffer was added up to a volume of 25 ml so that the line of the aqueous phase was at 25 ml and centrifuged for 10 min at 3500 rpm. 1.5 ml of the aqueous phase was pipetted into an Eppendorf tube and centrifuged in an Eppendorf centrifuge at 27,000/min for 10 min. The clear part of the solution was pipetted into an HPLC vial and used for liquid chromatography.

In order to check for cross contaminations during the sample pretreatment and to test the HPLC system suitability, at least one placebo suppository and one method blank sample were prepared for every sample batch or measurement sequence, respectively. In the case of method blank samples, neither Tonogen suppository nor placebo suppository was weighed in into the centrifuge tube, only the reagents used for the assay were used throughout the sample preparation process.

Measurement sequencies were immediately started after completion of the preparation of samples and calibration solutions. During a measurement sequence, samples were kept at stable 23 °C, the controlled ambient temperature of the laboratory. After completion of the sequence, samples were stored in a refrigerator at 56 °C.

Chromatographic conditions: (Table I).

At the beginning of a sequence, the method blank and the placebo samples were analysed. System suitability requirements comprised a stable retention time of adrenaline in the range of 4.3 ± 0.2 min and the absence of any adrenaline-disturbing chromatographic peak on the chromatograms of

Table I. Chromatographic conditions

Column	ACE Excel 3 μm C18-PFP 150×4.6 mm
Column temperature	30 °C
Elution technique	Gradient
Eluents	A: 50 mM KH_2PO_4 solution, pH=3.0
	B: Acetonitrile
Gradient	0 min 100% A
	2.0 min 100% A
	9.5 min 25% A – 75% B
	12.5 min 25% A – 75% B
	12.6 min 100% A
	15.5 min 100% A
Run time of measurement	15.5 min
Flow rate	1.0 ml/min
Injected volume	25 μl
Detector	UV 205 nm

Table II. Recovery of adrenaline from Tonogen+Peru balsam compounded rectal suppositories

	Sample name	Sample type	Mass of suppository	Quantity of spiked adrenaline	Adrenaline concentration in the sample	Adrenaline concentration RSD	Adrenaline content per suppository	Recovery of spiked adrenaline	Mean recovery of spiked adrenaline
			g	μg	μg/g	%	μg/suppository	%	%
	MethodBLANK	sample	*2.7**		*0.00*		*0.00*	-	
	MethodBLANK SP	spiked	*2.7**	33	*11.96*		*32.29*	97.8	
Placebo suppository	5101290/1 P1	sample	2.82		0.00		0.00	-	
	5101290/2 P2	sample	2.80		0.00		0.00	-	
	5101290/3 P3	sample	2.80		0.00		0.00	-	
Spiked placebo suppository	5101290/4 PSp1	spiked	2.81	33	11.95	0.87	33.58	101.8	100.4
	5101290/5 PSp2	spiked	2.79	33	11.75		32.77	99.3	
	5101290/6 PSp3	spiked	2.79	33	11.85		33.06	100.2	
Adrenaline suppository	5101291/1 M1	sample	2.73		10.51	6.12	28.69	-	
	5101291/2 M2	sample	2.73		10.58		28.89	-	
	5101291/3 M3	sample	2.76		9.47		26.13	-	
Spiked adrenaline suppository	5101291/4 MSp1	spiked	2.74	33	23.28	3.76	63.79	108.7	101.3
	5101291/5 MSp2	spiked	2.74	33	22.32		61.15	100.7	
	5101291/6 MSp3	spiked	2.73	33	21.60		58.98	94.4	

* Zero masses were replaced by the nominal mass of a suppository in order to perform calculations

the method blank and the placebo suppository sample, respectively.

Test of recovery prior to validation:

In order to investigate the recovery of adrenaline from the matrix, solutions were prepared from 3 placebo suppositories, 3 spiked placebo suppositories, 3 suppositories containing adrenaline, and 3 spiked suppositories containing adrenaline.

Preparation in the case of the spiked samples: after weighing in the sample, it was melted in a warm water bath, 33 μl of Tonogen solution was added, mixed and cooled back. Then a warmed buffer solution was added and the process is performed as above described.

For calibration, a series of calibration solutions from 1 mg/ml Tonogen solution was diluted in a concentration range of 0.220.0 μg/ml in 1-2-5 steps.

2.2 Validation of the assay method

Samples, chemicals and equipment:

Samples for the method validation:

The validation was performed using the 'placebo'

Table III. *Summary of the validation results of the HPLC-UV method*

Parameter	Requirement	Result
Specificity	No interference	**Fulfilled** in the ±1.2 min retention time range
Linearity	Linear according to the Mandel-test	**Fulfilled** in the 0.05-1.5 μg/ml range (appr. 5-135% adrenaline content)
	Reproducible	**Fulfilled** at P=0.95 (two independent calibrations compared)
	-	Correlation coefficients: r_1=0.99997, r_2=0.99981
	-	Coefficients of Variation: CV_1=0.54%, CV_2=1.46%
Quantitation limit (QL)	QL ≤ 0.6 μg/ml (test solution of a suppository with 50% adrenaline content)	**Fulfilled** QL = 0.2 μg/ml at *s*/*n* ≥10
Detection limit (DL)	-	DL = 0.1 μg/ml at *s*/*n* ≥5
Accuracy	Recovery between 90-110% in the range of 50-100% adrenaline content	**Fulfilled** at 10%, 50%, 100% adrenaline content: 98.1%, 99.7%, 98.8%, respectively
Repeatability	RSD ≤10% in the range of 50-100% adrenaline content	**Fulfilled** at 10%, 50%, 100% adrenaline content: 3.78%, 2.96%, 0.50%, respectively
Range of measurement	between 50-100% adrenaline content	**Fulfilled** between 10-100% adrenaline content

rectal suppositories prepared for the method development (Chapter 2.1).

Reference materials:

Adrenaline tartrate CRS (EDQM - Reference substances A0300000)

Noradrenaline tartrate CRS (EDQM - Reference substances N1100000)

Adrenaline impurity mixture CRS (EDQM - Reference substances Y0000740)

Adrenalone hydrochloride CRS (Supelco - Pharmaceutical Secondary Standard PHR2478)

Reagents (in addition to those listed in Chapter 2.1):

Hydrochloric acid fuming 37% (Merck EMSURE for analysis)

Solvents, solutions (in addition to those listed in Chapter 2.1):

Solvent 'B': 13 ml acetonitrile + 87 ml 50 mM KH_2PO_4 buffer

0.1 M HCl solution: 0.83 ml 37% Hydrochloric acid diluted to 100 ml with HPLC water

Adrenaline stock solution at 1 mg/ml: 18.2 mg Adrenaline tartrate CRS dissolved in 10 ml 50 mM KH_2PO_4 solution, pH=3.0 and stored at -18 °C.

Adrenaline working solutions at 100 μg/ml and 10 μg/ml: prepared by sequential dilution from the adrenaline stock solution with 50 mM KH_2PO_4 solution, pH=3.0 and stored at -18 °C.

Related substances: preparation of stock and working solutions of impurities B (noradrenaline), C (adrenalone), D and E (impurity mixture) was based on Ph Eur 9.0 [22] but adopted to the current chromatographic conditions using only 0.1 M HCl and 50 mM pH=3.0 KH_2PO_4 solutions without sodium octanesulfonate (ion pairing reagent) and stored at -18 °C.

Equipment (in addition to those listed in Chapter 2.1):

Analytical balance: Mettler Toledo AX205DR with 0.01 mg readability

Preparation of the samples for HPLC analysis:

The same procedure was applied as described in Chapter 2.1 including the spiking methodology described for the preliminary investigations of recovery. For the validation, the 1 mg/ml adrenaline stock solution, prepared from the CRS, was used as spiking solution instead of the Tonogen solution.

Methodology of validation:

The aim of the validation was to demonstrate that the test method is suitable for the determination of the nominal adrenaline content (up to 33 μg/suppository) of the Tonogen+Peru balsam compounded rectal suppositories and for the quantitative monitoring of the potential degradation of adrenaline up to a minimum of 50% content reduction with ≤10% relative error and ≤10% relative standard deviation.

The validation was performed according to ICH Q2(R2) guideline of 14 December 2023 [32]. As be-

Table IV. *Starting materials and their quantities used for the preparation of Tonogen+Peru balsam suppositories*

Starting materials	Theoretical quantity (g)	Batch number	Shelf life	Actual weighed in quantity (g)
Benzocaine Ph. Eur. 10.	20	2309-4340	07. 03. 2025.	20 Recovered after pulverization: 19.9
Peru balsam Ph. Eur.10.	8	2310-4376	11. 02. 2027.	8.1
Tonogen amp. 1mg/ml	2 ampoules	A24092	04. 2026.	2 ampoules (2000 μg)
Adeps Solidus Ph. Eur. 11.	158.4	2305-2061	07. 06. 2026.	158.4
Ricini oleum virginale Ph. Eur. 11.	as needed	2402-4113	31. 12. 2025.	12.5
Total quantity of bulk suppository mass:				200.9 g
Nominal average adrenaline concentration in the bulk suppository mass:				**9.96 μg/g**
Quantity of leftover (leftover removed from the suppository mould):				6.5 g
Calculated total mass of the manufactured 68 suppositories:				194.4 g
Calculated average mass of individual suppositories:				2.86 g

ing a content quantitation-type investigation, the performance characteristics Specificity, Range, Accuracy and Precision were validated (see Annex 1 and Annex 2 of the guideline). Robustness was not investigated. The actual validation tests and the necessary solutions are listed as follows:

- *To check Specificity, the 'Absence of Interference' was validated using prepared solutions of a method blank, a placebo suppository and a spiked placebo suppository, and diluted solutions of the known impurities B, C, D and E as well, respectively.*
- *To check Range, 'Validation of Calibration Model' (linearity) and 'Validation of Range Limits' (quantitation limit – QL, detection limit – DL) were performed. For these purposes, high-resolution, 12-point calibration series were prepared from the adrenaline working solutions in two independent replicates at the following concentration levels: 0.05, 0.1, 0.2, 0.3, 0.45, 0.6, 0.75, 0.9, 1.05, 1.2, 1.35, 1.5 μg/ml. This calibration range corresponds 1.2537.5 μg adrenaline per suppository, that means a range of approximately 5135% adrenaline content when an adrenaline concentration of 1010.5 μg/g in the bulk mass and a suppository mass of 2.72.8 g are assumed.*
- *Accuracy was investigated by 'Spiking Study': a method blank, placebo samples in 3 replicate preparations and spiked placebo samples at the calculated drug content, 50% drug content and 10% drug content levels in 6 to 6 replicate preparations were assayed. For this test, a calibration series of 5 levels was prepared from the adrenaline working solutions at 0.1, 0.3, 0.6, 0.9, 1.2 μg/ml. This calibration range corresponds approximately 10110% adrenaline content.*
- *Precision was characterized as 'Repeatability', by evaluating the results of Accuracy test.*

The ChemStation (Agilent) software was used for quantitative evaluation of the results and statistical calculations were performed in Excel (Microsoft).

2.3 Stability study

Samples:

For the stability study, 68 pcs individually numbered Tonogen+Peru balsam compounded rectal suppositories were moulded under the following conditions:

Laboratory air temperature: 23 °C

Temperature of the melted adeps solidus: 37.8 °C (start of heating: 10 h 10 min am.)

Temperature of the liquid suppository mass immediately before addition of the adrenaline solution: 30.8 °C.

Starting time for moulding the first 30 suppositories: 10 h 45 min.

Starting time for moulding the second 30 suppositories: 10 h 49 min.

Time for placing the moulded suppositories in the refrigerator: 10 h 56 min.

The starting materials and their quantities used for suppository preparation are described in **Table IV**.

Storage conditions and experimental design:

Storage conditions: During the stability test, suppository samples were stored in a refrigerator, the temperature of which was checked 3 times a week (Monday, Wednesday, Friday) with a thermometer. The temperature of the refrigerator fluctuated between 5.0-6.0 °C during the 28 days of storage.

Experimental design: Subsets of the suppository samples were analysed on the day of preparation (t0) and after 1, 2 and 4 weeks of storage (t1, t2, t3), respectively. Detailed explanations on the grouping of the samples into the subsets will be given in Chapter 3.3 and 3.4.

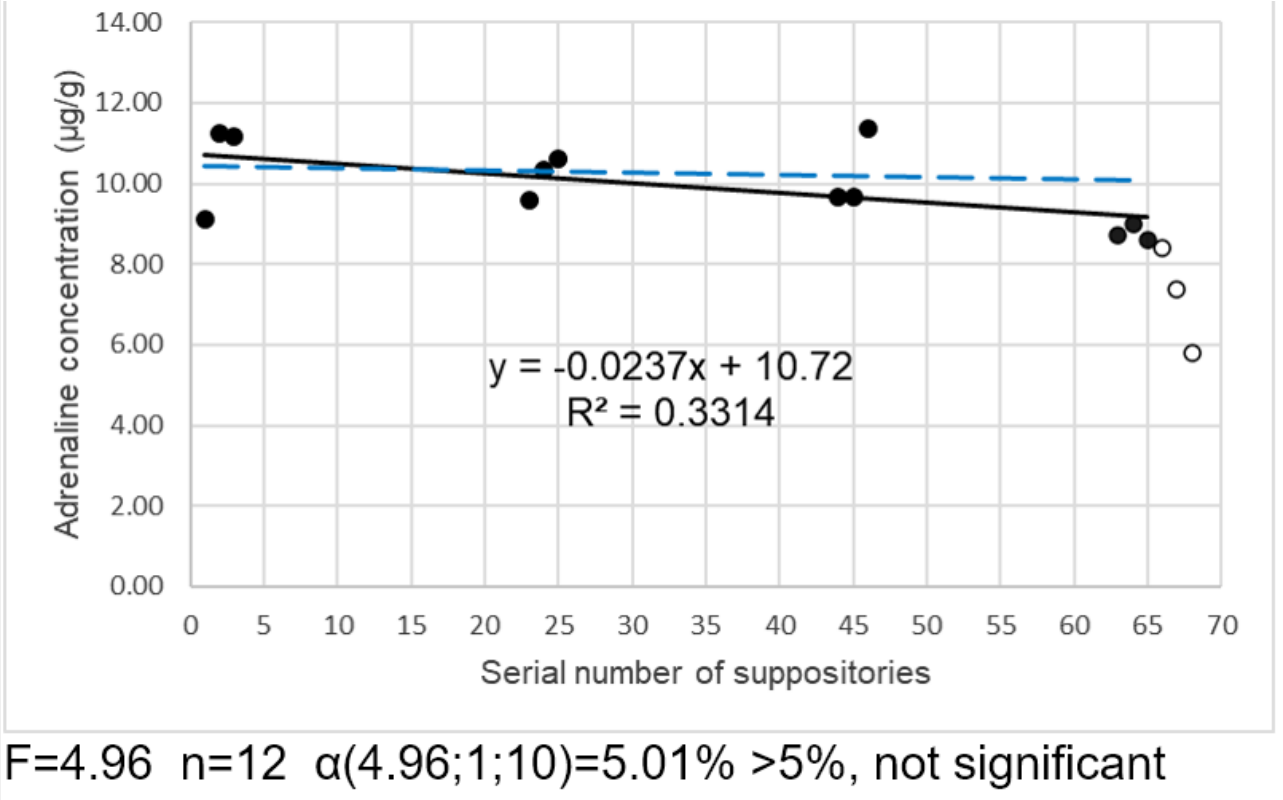


Figure 1 *Typical HPLC-UV chromatogram from a Tonogen+Peru balsam rectal suppository at 205 nm (blue), 210 nm (red), and 280 nm (green) wavelengths using the described method*

The samples were prepared for analysis and assayed by HPLC as described in Chapter 2.1, using 5-level calibration solutions as described for the validation of Accuracy in Chapter 2.2. In every measurement sequence performed for the stability study, the full calibration series was analysed twice, at the beginning and at the end of the sequence as well, in order to check for analyte decomposition in the sample vials and instrumental drift, respectively. As we always observed reproducible peak areas, all calibration data could be combined into a single, 10-point calibration curve for each measurement sequence.

3. Results

3.1 Development of the assay method and investigation of the recovery

At the beginning of the method development, our first decision was that we try to solve the separation of adrenaline without the use of ion-pair chromatography when possible. Although Ph. Eur. still specify IP-HPLC for the related substances of adrenaline [22, 23], a lot of alternative chromatographic solutions have been published meanwhile [26, 27, 28, 29, 30] that offer at least two benefits over IP-HPLC: the lack of ion-pairing reagent lowers the cost of analyses significantly and simplifies the preparation of eluents. The latter has a further impact on the robustness by eliminating the risk of failure due to improper dosage of the IP-reagent.

Among the reviewed papers and application notes, the pentafluorophenylpropyl-phase (PVP) appeared to promise the highest retention for adrenaline, showing a retention factor of approximately k=1.9, using the described phase system [30]. The superior retention for adrenaline is due to the pentafluorophenyl functional groups spaced by short propyl-chains from the silica surface of the stationary phase. Finally, we decided to use another stationary phase, the ACE Excel 3 µm C18-PFP-phase, because it exhibits similar retention mechanism for adrenaline but also provides even higher retention for the huge amounts of the less polar matrix compounds due to its C18-chains [33].

Detailed description of the optimisation of the chromatographic conditions is beyond the scope of the present paper. In brief, phosphate puffer and acetonitrile were chosen for the eluent in fa-

Table V. *Division of the individually numbered Tonogen+Peru balsam rectal suppositories for testing at time t0*

Manufacture of suppositories	Serial numbers of suppositories						
Beginning (6 pcs): t0 and trend	1-6.						
1st third (15 pcs): for stability study		7-21.					
between the 1st-2nd thirds (6 pcs): t0 and trend			22-27.				
2nd third (14 pcs): for stability study				28-41.			
between the 2nd-3rd thirds (6 pcs): t0 and trend					42-47.		
3rd third (15 pcs): for stability study						48-62.	
End (6 pcs): t0 and trend							63-68.

vour of maximum sensitivity at low wavelength detections (205 nm). The phosphate puffer concentration of 50 mM, the pH-value=3 and the column temperature of 30 °C represent a good compromise between chromatographic peak shape and long-term use of the column without significant wear-off. Figure 1. presents a typical HPLC-UV chromatogram from a Tonogen+Peru balsam rectal suppository in which the adrenaline peak is clearly separated at a retention time of 4.29 min.

The data in Table 2 show that the 'placebo' suppositories, by definition, do not contain detectable amounts of adrenaline. From the 33 μg adrenaline-spiked placebos, we were able to measure the concentration of added adrenaline with an RSD of 0.87%. The mean recovery rate of added adrenaline was 100.4%.

In the suppositories with adrenaline, the concentration of prepared adrenaline could be determined with an RSD of 6.12% and we were able to measure the calculated nominal average value (27.8 μg/suppository) based on the weigh-in masses: mean: 27.9 μg/suppository; min: 26.13 μg/suppository; max: 28.89 μg/suppository. These results indicate that the preparation process of the suppositories appears to cause no major degradation of adrenaline. On the other hand, the difference between the RSDs of spiked and prepared adrenaline concentrations, 0.87% vs. 6.12%, respectively, reveals some differences in the distribution of adrenaline in the suppositories i. e. inhomogeneity in the bulk mass.

In the 33 μg adrenaline-spiked suppositories we measured the total concentration of adrenaline with an RSD of 3.76%. Single recoveries were calculated as the difference of the measured total adrenaline concentration and the mean of the measured prepared adrenaline concentrations of the non-spiked sample set (10.2 μg/g), then multiplied by the actual suppository mass and divided by the added amount of 33 μg adrenaline. The mean recovery rate of spiked adrenaline was 101.3%. (**Table II.**)

3.2 Validation of the assay method

Detailed presentation of the validation is beyond the scope of the present paper, only a brief summary of the results is given in this chapter. The Specificity was proved as no interfering peaks could be detected on the chromatograms of the method blank, the placebo suppository and the known impurities B, C, D, E in the retention time window between 35.5 min, similarly to the chromatogram depicted on Figure 1.

The results are shown in **Table III**. The HPLC-UV method is sufficiently specific, linear, sensitive, accurate and precise according to the criteria previously defined and beyond, making it suitable for the determination of adrenaline from Tonogen+Peru balsam rectal suppository in the range of 10-100% of the nominal drug content. Based on the results, the method is considered validated. (**Table III.**)

3.3 Characteristics of Tonogen+Peru balsam suppositories at t0 time point

The individually numbered suppositories were divided into 3 major groups, representing the first, middle and last third part of the suppository preparation process, and into 4 minor groups with suppositories produced at the borders of the three major groups mentioned above, i.e. at the very beginning of the suppository preparation, between the first and middle third parts, between the middle and third part, and at the very end. The divisions are illustrated in **Table V**.

From the four 't0 and trend' groups, 3 to 3 suppositories were selected (n=12) to test their mass and adrenaline content, so that the suppository manufacturing process was represented in a uniform manner from the first to the last suppository: suppositories Nr 1-3; Nr 23-25; Nr 44-46 and Nr 66-68. The remaining 3-3 suppositories of the groups were left in reserve.

Results of the adrenaline assay are presented in

Table VI. *Results of the adrenaline assay from suppositories of the "t0 and trend" groups (see Table 5.)*

Serial number of suppositories	Mass of suppository (g)	Adrenaline concentration	
		(μg/g)	Compared to the nominal average conc. in bulk mass (9.96 μg/g)
1.	2.72	9.11	92%
2.	2.77	11.24	113%
3.	2.76	11.16	112%
23.	2.77	9.61	96%
24.	2.76	10.35	104%
25.	2.75	10.62	107%
44.	2.78	9.65	97%
45.	2.78	9.65	97%
46.	2.77	11.35	114%
66.	2.79	8.41	84%
67.	2.81	7.36	74%
68.	2.81	5.80	58%
63. (after 1 day, t0′)	2.79	8.72	88%
64. (after 1 day, t0′)	2.80	9.01	90%
65. (after 1 day, t0′)	2.78	8.60	86%

Table VI. The system suitability requirements, as listed in Chapter 2.1 *Chromatographic conditions*, were fulfilled. Moreover, the duplicate calibration measurements, at the beginning and the end of the measurement sequence, were also reproducible. This indicates stable sample solutions and instrumental conditions. The fulfilment of the system suitability criteria and the stable analytical conditions applied also for the sequencies at t1, t2 and t3 time points whose results will be discussed later in Chapter 3.4. (**Table VI.**)

The results show that the mass of the supposi-

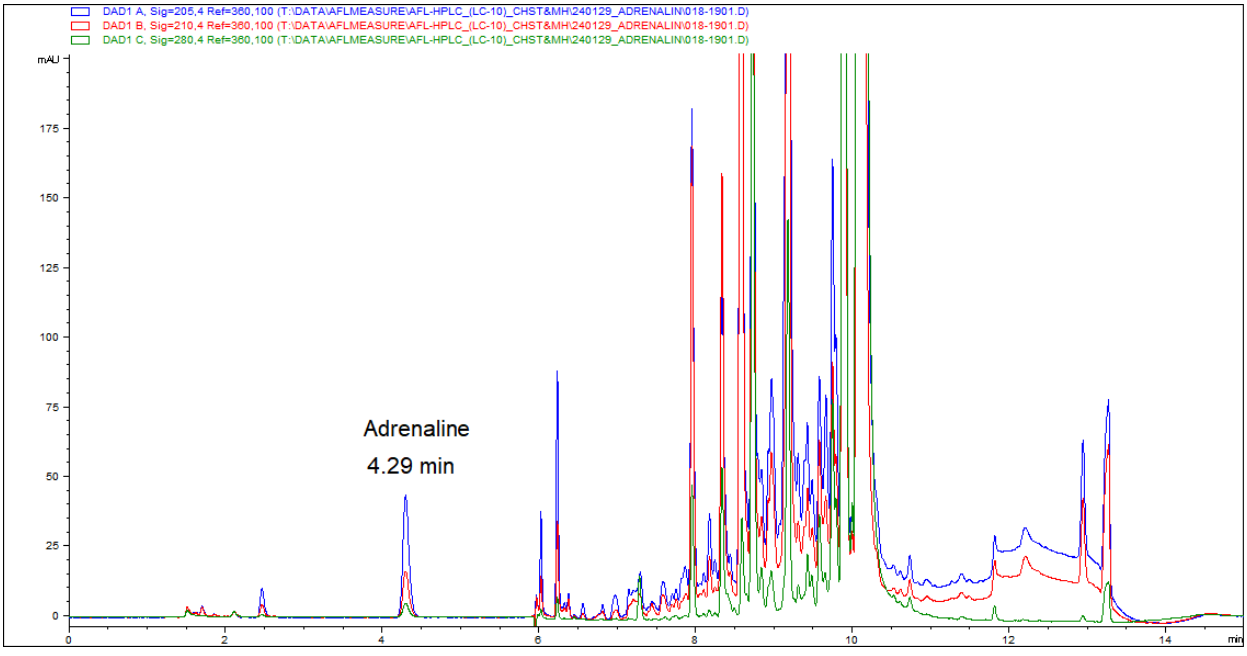


Figure 2 *Parameters of lines that can be fitted to rectal suppository mass data and to adrenaline concentration data, respectively, versus serial number of the suppositories*

tories increases slightly, while the adrenaline concentration per suppository mass decreases during the suppository manufacturing (from 1 to 68). The parameters of the fitted lines are shown in **Figure 2** (in black).

In Figure 2, the F-values of the fits are also given, from which the α-values can be calculated with (1;n-2) degrees of freedom. High F-values and accordingly low α-values indicate a significant relationship between the dependent variable (suppository mass or adrenaline concentration) and the independent variable (serial number of the suppositories). For α<5%, there is a P>95% probability of a significant relationship. In both plotted curves, the correlation is significant, as indicated by the statistical measures of the line fitting to the data series. The variation in suppository weight remains significant even when the first suppository (m=2.72 g) is removed from the model. However, as the main focus of the study has been the stability of adrenaline, and the concentration of adrenaline has always been given in terms of the mass of the tested suppository, the change in the mass of the suppositories and its significance are not discussed further.

There was a marked decrease of adrenaline concentration for the last 3 suppositories tested (84%, 74%, 58%, respectively, relative to the nominal average in the bulk suppository mass). Without these values, the line fitted to the first 9 points shows no significant change. The question is whether the concentration of adrenaline decreases steadily in the last third of manufacturing, or whether it breaks down abruptly at the very end. Deciding this question has a bearing on whether the suppositories manufactured in the last third are representative enough to be considered in the stability test. To decide this, we also tested suppositories Nr. 63-64-65 remaining in the reserve 1 day after manufacturing, the results of which are shown at the end of Table 6. Graphically, **Figure 3** illustrates the results.

It can be concluded that of the last low adrenaline concentrations measured in suppositories Nr. 66-67-68 (which are now not included in the model), only the last two can be identified as 'sudden breakdown'. However, the result of suppository Nr. 66 is confirmed by the similarly low concentrations measured in suppositories Nr. 63-64-65. These suggest a concentration decline in the last third of production, the profile of which is uncertain.

On the other hand, from a purely statistical point of view, the decrease in adrenaline concentration between suppositories Nr. 1 and Nr. 65 cannot be considered significant, i.e. no trend can be statistically detected, although the decision criterion is borderline (5.01% vs. 5%). Based on these results, and on a purely statistical judgement, the suppositories produced in the last third can also be assumed to be representative.

3.4 Results of a 28-day stability study of Tonogen+Peru balsam compounded rectal suppositories

Following the measurements at time t0, the remaining spare suppositories from the t0 and trend groups were added to the larger groups representing three thirds of the production as shown in **Table VII**.

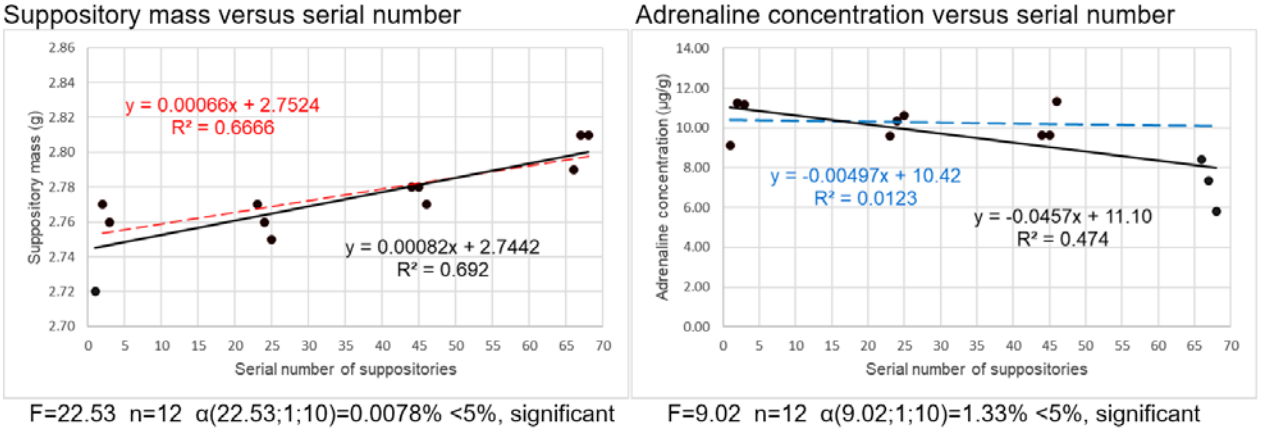


Figure 3 *The adrenaline content as a function of the serial number of rectal suppositories*

Table VII *Grouping of individually numbered Tonogen+Peru balsam compounded rectal suppositories for stability testing*

Manufacture of suppositories	Serial numbers of suppositories		
1st third (19 pcs): stability study (and trend)	4-22.		
2nd third (18 pcs): stability study (and trend)		26-43.	
3rd third (16 pcs): stability study (and trend)			47-62.

Serial No. of suppository	Mass of suppository (g)	Adrenaline conc. (µg/g)	comp. to 9.96 µg/g*
4.	2.76	10.55	106%
11.	2.76	9.51	95%
22.	2.75	9.36	94%
27.	2.77	9.87	99%
35.	2.82	10.26	103%
41.	2.76	7.64	77%
48.	2.75	10.20	102%
54.	2.77	9.28	93%
58.	2.80	9.05	91%

*Nominal average adrenaline conc. in the bulk mass

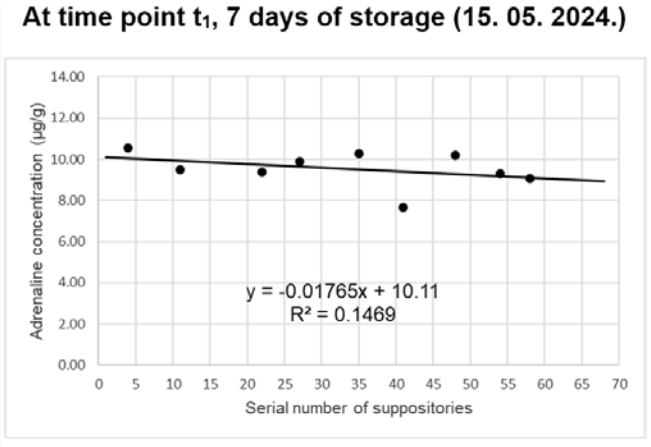


Figure 4 *Adrenaline stability results after 7 days of storage*

Table VIII *Numerical values of adrenaline concentrations in the Tonogen+Peru balsam compounded rectal suppository stability study from t0 to t3 (week 4)*

Time point	Adrenaline concentration				
	mean	standard deviation	rel. standard deviation	mean	standard deviation
	µg/g			compared to the nominal average conc. in bulk mass (9.96 µg/g)	
t0 and t0', start	9.92	0.99	10.0%	99.7%	10%
t1, 1st week	9.52	0.87	9.1%	95.7%	8.7%
t2, 2nd week	9.15	0.60	6.6%	91.9%	6.0%
t3, 4th week	9.91	0.76	7.6%	99.6%	7.6%

Stability results had to be obtained from at least 6 parallel measurements at each time point. Given the uncertainty about the last third of production, the following experimental design was adopted: 3-3 randomly selected samples from each of the three thirds of production (n=9) were tested at each storage time point, and for each storage time point, a trend was tested separately. In the light of the results, we decided whether to consider all 9 measurements or only the 6 results from the first two thirds. The t0' results of suppositories Nr 63-64-65 were taken into account for the evaluation of the results instead of the t0 results of suppositories Nr 66-67-68.

The results are presented in **Figures 4, 5** and **6**.

No trend has been observed at any of the storage times in relation to the order of manufacturing, so the results of both the 12 (9 t0 + 3 t0') and the 9-9-9 (t1, t2, t3) parallel measurements have been included in the final stability test results.

A summary of the stability test results is shown in **Table VIII**.

4. Discussion, conclusions

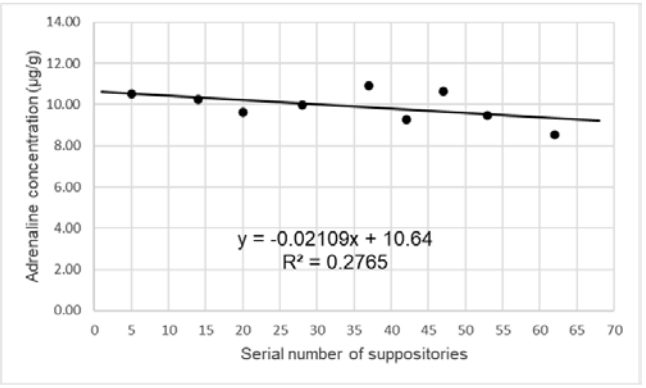
The most important conclusion from the results is that during the 28-day refrigerated storage period (5.0-6.0 °C), the mean concentration of adrenaline in Tonogen+Peru balsam compounded rectal suppositories did not show a significant decrease.

However, significant differences were found between the adrenaline content of the individual suppositories: the two last suppositories of the manufacturing process contained only 74% and 58% of the nominal average bulk concentration at the time of production (t0), respectively. Within

Serial No. of suppository	Mass of suppository (g)	Adrenaline conc. (µg/g)	Adrenaline conc. comp. to 9.96 µg/g*
5.	2.75	10.51	106%
14.	2.77	10.27	103%
20.	2.74	9.63	97%
28.	2.78	9.99	100%
37.	2.72	10.90	110%
42.	2.75	9.26	93%
47.	2.76	10.65	107%
53.	2.76	9.47	95%
62.	2.78	8.54	86%

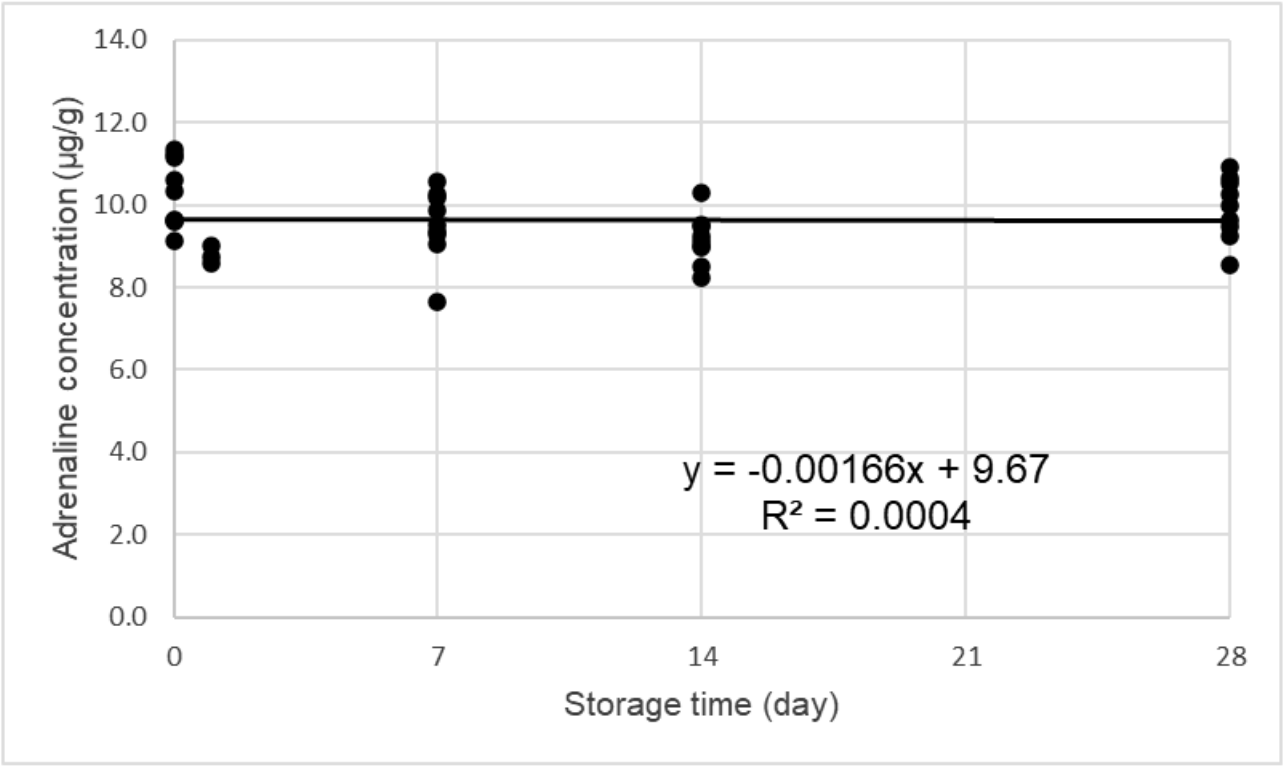
*Nominal average adrenaline conc. in the bulk mass

At time point t_3, 28 days of storage (05. 06. 2024.)



F=2.67 n=9 α(2.67;1;7)=15% >5%, not significant

Figure 6 *Adrenaline stability results after 28 days of storage*



F=0.015 n=39 α(0.015;1;37)=90% >5%, not significant

Figure 7 *Adrenaline contents from the manufacturing range Nrs 1-65 suppositories plotted individually as a function of storage time*

the population finally considered in the stability study, the lowest measured concentration (suppository Nr. 41; 7.64 µg/g) was 77% of the nominal average bulk concentration at t1, while the highest measured concentration (suppository Nr. 46; 11.35 µg/g) was 114% of the nominal average bulk concentration at t0. The difference between the two values is greater than 30%, indicating that a large number of suppositories need to be tested in parallel to obtain reliable mean values.

With the 12- and 9-suppository populations tested, we just managed to remain within 10% relative standard deviation for each storage time. The inhomogeneity found in the population does not show a significant trend as a function of the number of suppositories manufactured (if the last

suppositories manufactured were excluded; see results in previous chapters).

Plotted individually as a function of storage time, the adrenaline contents of the suppositories tested from the manufacturing range Nr 1 to Nr 65 suppositories is fitted by the line as shown in Figure 7; practically no decrease was observed.

Adrenaline concentrations other than the nominal are more likely to be due to the characteristics of the compounded manufacturing of rectal suppositories, especially in the case of low adrenaline concentrations in suppositories prepared at the very end of the manufacturing process.

This work is the first one in the international literature on the assay and stability testing of adrenaline from rectal suppositories, specifically from suppositories with a complex active substance composition.

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