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Multi-residue method for non-steroidal anti-inflammatory drugs in plasma using high-performance liquid chromatography–photodiode-array detection
Method description and comprehensive in-house validation

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Abstract

A multi-residue high-performance liquid chromatography (HPLC) method with photodiode-array detection is presented for the determination of 12 non-steroidal anti-inflammatory drugs (NSAIDs) in plasma. This method has been validated under the consideration of actual α- and β-errors according to an in-house validation concept based on a fractional factorial experiment. A wide range of matrices and other influencing factors have been included in the validation experiment. In order to assess the method's performance the power curve, which demonstrates the detection power of the analytical method, was computed and CC_α and CC_β values were calculated. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Non-steroidal anti-inflammatory drugs

1. Introduction

NSAIDs (non-steroidal anti-inflammatory drugs) are widely used in human and veterinary medicine for their ability to either suppress or reduce the inflammatory process and the clinical signs associated with it, such as heat, pain, swelling, hyperaemia and loss of function.

NSAIDs represent an heterogeneous group of compounds (Fig. 1). They are often chemically unrelated, although most of them are organic acids. The prototype is aspirin; hence these compounds are often referred to as aspirin-like drugs. The following classes of compounds can be distinguished: (1) salicylic acid derivatives; (2) propionic acid derivatives; (3) pyrazole derivatives and (4) aniline derivatives including nicotinic acid and anthranilic acid derivatives.

All aspirin-like drugs are antipyretic, analgesic and anti-inflammatory, but there are important differences in their activity. Some are not suitable for either routine or prolonged use because of their toxicity, e.g. phenylbutazone. The most common side-effect is a propensity to induce gastric or

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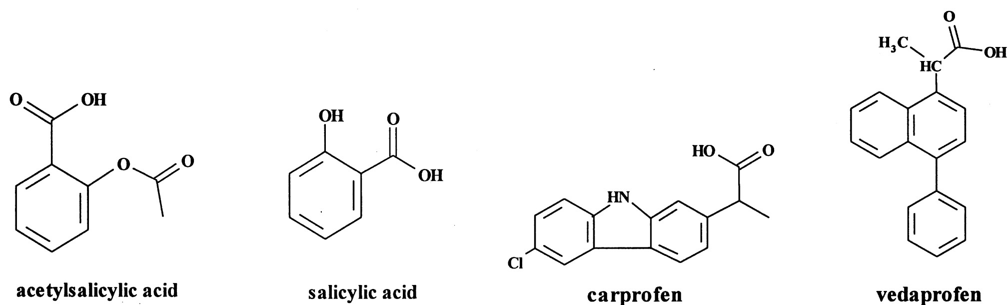
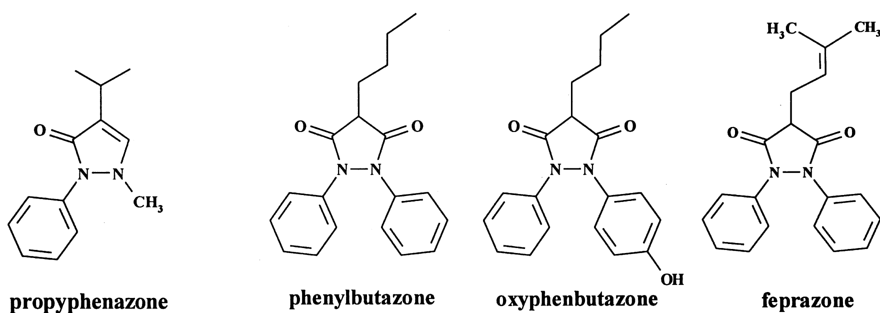
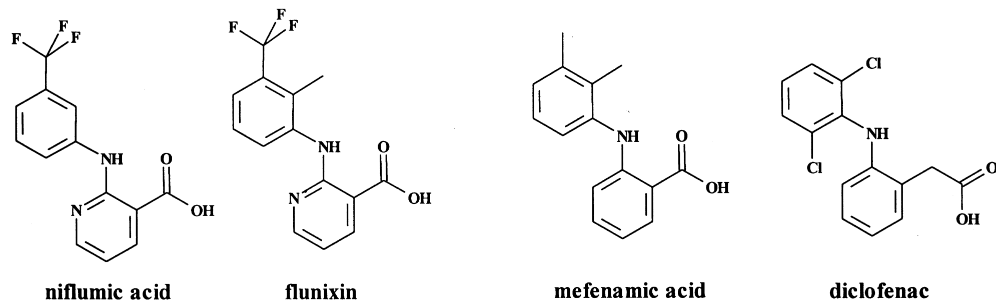
Molecular structures of the examined NSAIDs



Salicylic acid derivatives **Propionic acid derivatives**



Pyrazole derivatives



Nicotinic acid derivatives **Anthranilic acid derivatives**

Fig. 1. Molecular structures of the investigated NSAIDs.

intestinal ulceration that can sometimes be accompanied by anaemia from the resultant blood loss. Other side-effects include disturbances in platelet function, the prolongation of gestation or spontaneous labour, and changes in renal function [1]. Long-term exposure to phenylbutazone induces kidney tumours in rats and liver tumours in mice [2].

To protect consumers from health-threatening residues of veterinary drugs and their metabolites, in the European Union following the provisions of Council Regulation (EEC) 2377/90 [3] each pharmacologically active substance administered to food-producing animals must be assigned a maximum residue limit (MRL).

Although the MRL procedure has not yet been completed for most of the NSAIDs, it seemed reasonable to develop a multi-residue method including as many NSAIDs as possible in order to have the possibility of comprehensive control. As it can rightly be assumed that a permanent ban might be imposed on certain NSAIDs (e.g., phenylbutazone) requiring more stringent control of a possible misuse of these substances in live animals, plasma was chosen as the matrix to be investigated.

Thus far NSAIDs have been subject to many investigations such as multi- and single-compound HPLC methods and analytical methods for the detection of analytes in various matrices, especially in plasma or urine [4–10]; even liquid chromatography–mass spectrometry (LC–MS) methods are described [11]. The present study particularly emphasizes the comprehensive validation of the method described. For the first time the newly developed in-house validation concept [12,13] was applied to a multi-residue method.

The validation experiment was based on a fractional scheme considering the very important matrix- and time-induced deviations, and the random measurement error in a single experiment. In applying this scheme very important components of errors are considered that possibly influence analytical measurement results in residue and trace analysis.

By applying the in-house validation concept it is possible to calculate the performance parameters CC_α and CC_β (see *Definitions*). The performance of the validated method is demonstrated by the power curve as well as by the overall and the matrix-specific calibration functions.

2. Experimental

2.1. Reagents and materials

Unless indicated otherwise, analytically pure substances and HPLC-grade solvents were used. Acetylsalicylic acid (ASS) [50-78-2] 99.7%, salicylic acid (SA) [69-72-7] 99.0%, oxyphenbutazone hydrate (OPB) [7081-38-1] 1 mol/mol, carprofen (CPF) [53716-49-7], niflumic acid (NFA) [4394-00-7], diclofenac, sodium (DC) [15307-79-6], phenylbutazone (PBZ) [50-33-9], mefenamic acid (MFAS) [61-68-7], and ketoprofen (KTP) [22071-15-4] were purchased from Sigma (St. Louis, MO, USA). Propyphenazone (PPHZ) [479-92-5] was a gift from Berlin-Chemie AG (Berlin, Germany) 99.98%. Flunixin meglumine (FLU) [38677-85-9] was a gift from Schering Plough (Segrè, France) 99.3%. Vedaprofen (VDP) [71109-09-6] was a gift from Intervet International B.V. (Boxmer, The Netherlands) 99.0%. Hexane, methanol, acetonitrile and water were obtained from Fisher Scientific (Wiesbaden, Germany). Diethyl ether was purchased from Merck (Darmstadt, Germany). Hydrochloric acid (1 *M*, titrisol quality), sodium chloride and acetic acid (1 *M*, titrisol quality) were from Merck. Ascorbic acid was from Sigma. C_{18} octadecyl LRC (filling mass 500 mg) cartridges were obtained from Baker (Griesheim, Germany). Ascorbic acid solution was produced by dilution of 0.01 mol ascorbic acid salt with 1 l H_2O. Acetic acid solution was produced by dilution of 250 ml 1 *M* acetic acid solution with 2250 ml H_2O. The concentration of this solution was 0.1 *M* with a pH of 3.0. The standard solutions were produced as follows. Dilution of all standard solutions was carried out by using an acetonitrile–methanol mixture 9+1. The NSAID-S0 solution was prepared by accurately weighing 10.0±0.05 mg of each standard substance and adding exactly 10 ml of acetonitrile containing 10% methanol. The solution was then placed in an ultrasonic bath and swirled until the substances dissolved. The concentration of this solution was 1 mg ml^{-1}. Due to the dissolving process in the case of OPB, FLU and DC the free acids were present instead of the hydrate or salt. Therefore, the calculated concentration had to be multiplied by a correction factor: $f_{(OPB)}=0.947$; $f_{(FLU)}=0.603$; $f_{(DC)}=0.931$. NSAID-S1 (100

μg ml^{-1}), S2 (10 μg ml^{-1}) and S3 (1 μg ml^{-1}) were produced by diluting appropriate volumes of the NSAID-S0 stock solution with acetonitrile containing 10% methanol. The standard solutions were refrigerated at −20°C. When stored in the freezer, these standard solutions are stable for at least 6 months. Further dilutions were always carried out using acetonitrile–methanol mixtures (9+1).

2.2. HPLC conditions

A Hewlett-Packard Series II 1090 liquid chromatograph with photodiode-array detector (Waldbronn, Germany) was used. This chromatograph is equipped with a ternary solvent delivery system with low pressure pre-pumps and a high pressure mixing chamber for efficient and reproducible mixing of solvent proportions. The software offered the possibility of library search tests as well as peak purity tests. An ODS II Inertsil (250×4.6 mm, 5 μm) column connected to a pre-column (ODS II Inertsil, 8 mm, 5 μm) was conditioned with the starting composition of the gradient. Gradient A, acetic acid solution; gradient B, acetonitrile. A linear gradient was applied starting with 2 min 80% A, followed by a linear gradient to 36% A at 25 min and going back to the starting conditions over 5 min. The equilibration time was 10 min. An oven tem-

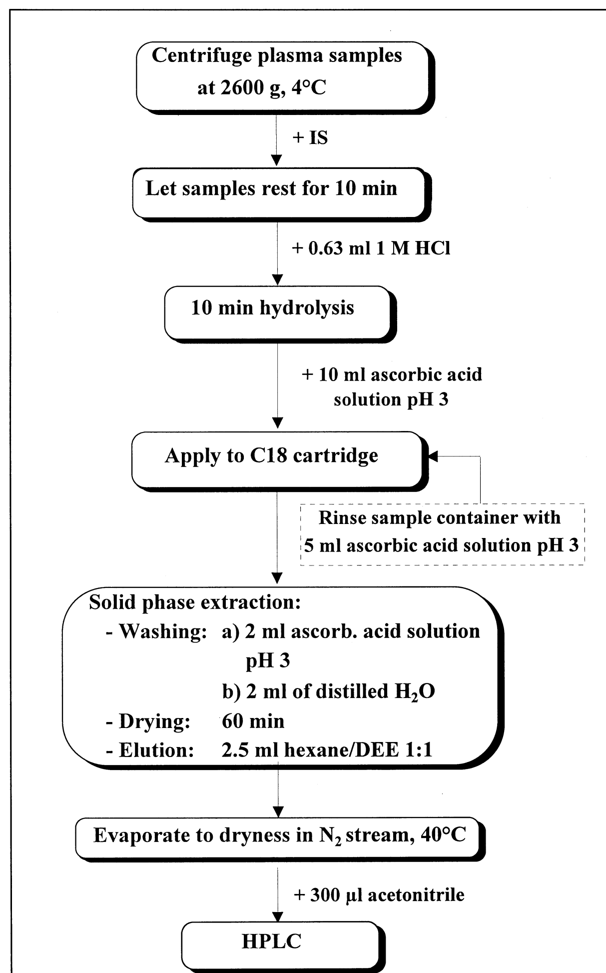


Fig. 2. Sample pre-treatment scheme for the extraction of NSAIDs from plasma.

perature of 40°C, a flow-rate of 1 ml min^{-1}, an injection volume of 10 μl and an autosampler rack temperature of 8°C were used. The monitoring wavelengths were 240, 278 and 290 nm. A peak-controlled spectrum recording was selected with a range of 240–400 nm and a step width of 2 nm. The system was able to produce a signal-to-noise ratio of 3:1 when injecting 10 μl of a 0.5 ng μl^{-1} phenylbutazone standard solution.

2.3. Procedure

Heparin-treated syringes and flasks were used for taking the blood samples. To isolate the plasma, the blood was centrifuged at 2600 *g* and 4°C. The laboratory sample was divided into subsamples of approximately 50 ml. The subsamples were stored in a freezer at approximately −20°C. After defrosting, the samples had to be centrifuged again at 2600 *g* and 4°C since decomposition processes begin quickly even in deep-frozen samples, noticeable by a change of colour and precipitation of matrix particles after defrosting. (After 2 weeks of storage at −25°C a change in the plasma was noticed irrespective of the species, whereas no change was noticed after 1 day of storage at −25°C.) The frozen subsamples were thawed in a water bath at 20°C. Alternatively, the subsamples can also be thawed overnight in the refrigerator at about 4°C.

Table 1
Concentration ranges of the standard calibration curves used in the validation experiment

Absolute analyte amount on column	Standard concentrations (ng μl^{-1})
I: 1–11 ng	0.1
	0.3
	0.5
	0.7
	0.9
	1.1
II: 10–110 ng	1
	3
	5
	7
	9
	11
III: 100–600 ng	10
	20
	30
	40
	50
IV: 1000–2200 ng	100
	120
	140
	160
	180
	200
	220

The spike experiments for the validation experiment were performed by adding the standard solution to the test sample directly after the centrifugation step following the thawing process.

After the samples had been treated as described, 5 ml plasma was pipetted into 47.5 ml plastic centrifuge tubules. (During the validation experiment it could be shown that the test sample volume can be varied between 0.5 and 6 ml. The dilution volumes were adapted accordingly.) The plasma was adjusted to pH 3 by adding 1 *M* HCl (approximately 0.63 ml) and was then hydrolysed at room temperature for 10 min. (No longer; risk of decomposition!) After waiting for 10 min and subsequent dilution with 15 ml 0.01 *M* ascorbic acid solution (pH 3), the solution was applied to the cleaned (2 ml hexane–diethyl ether, 1+1), preconditioned (2 ml methanol) and equilibrated (2 ml ascorbic acid solution, pH 3) C_{18} cartridge. Using Pasteur pipettes the diluted plasma was applied quantitatively to the solid-phase cartridges while the sample container was rinsed with approximately 5 ml pH 3 ascorbic acid solution. (The number of rinsing steps and the solvent volumes have to be adjusted to the viscosity of the matrix and depend on the initial matrix volume.) To rinse the cartridges, 2 ml pH 3 ascorbic acid solution, followed by 2 ml water were applied. Afterwards, the cartridges were dried at full water-jet pump vacuum for approx. 60 min. The analytes were eluted with a mixture of 2.5 ml hexane–diethyl ether (1+1). The eluate was evaporated to dryness in a nitrogen stream at 40°C in the evaporation station and then reconstituted in 300 μl acetonitrile containing 10% methanol (Fig. 2).

3. Validation experiment

The validation experiment was performed using the plasma of five different species: calves, bovines, ovines, porcines and equines. Different feeding

Table 2
Regression parameters of the external standard calibration curves: (I) 1–11 ng; (II) 10–110 ng; (III) 100–600 ng; (IV) 1000–2200 ng (see also Table 1)

Substance	Intercept a				Slope b			
	I	II	III	IV	I	II	III	IV
SA	−0.444	0.381	2.45	−8.88	0.568	0.590	0.576	0.645
OPB	0.213	0.100	1.091	−0.0463	0.256	0.323	0.322	0.337
FLU	0.178	0.149	0.0858	−0.514	0.618	0.645	0.647	0.678
CPF	0.0593	0.272	2.982	13.28	1.45	1.42	1.45	1.46
DC	−0.233	0.394	2.089	−0.0596	1.23	1.23	1.23	1.25
NFA	0.523	0.697	2.577	−4.73	2.68	2.63	2.64	2.71
PBZ	0.0782	−0.627	2.733	−0.875	1.45	1.61	1.60	1.66
MFAS	−0.0816	0.219	2.036	3.56	0.994	0.990	1.02	1.04
VDP	0.0840	0.630	1.758	−2.92	0.952	0.972	0.990	1.00
	Correlation coefficient				Residual standard deviation			
	I	II	III	IV	I	II	III	IV
SA	0.998	0.9990	0.997	0.9994	0.14316	0.89571	3.09931	17.73061
OPB	0.991	0.9996	0.9996	0.9993	0.15082	0.40887	2.17001	9.26119
FLU	0.997	0.9996	0.9998	0.9993	0.20178	0.87182	2.92124	18.96049
CPF	0.999	0.9998	0.9998	0.9994	0.33420	1.38296	7.53746	39.13989
DC	0.998	0.9997	0.9998	0.9994	0.33708	1.33782	6.48645	32.05110
NFA	0.999	0.9998	0.9998	0.9994	0.47045	2.33270	13.34700	68.49904
PBZ	0.999	0.9998	0.9998	0.9994	0.33492	1.45946	8.51215	42.40661
MFAS	0.998	0.9998	0.9998	0.9992	0.29549	0.88757	5.03260	30.40466
VDP	0.998	0.9997	0.9998	0.9994	0.29941	1.08602	4.97156	26.13791

conditions (intensive, extensive), different sampling (transportation, centrifugation) and storing conditions (1–4 days, several weeks), different thawing processes (0.5 h at 20°C, 16–20 h at 4°C), different matrix volumes per concentration and operators with different experiences on two factor levels were taken into account when planning the experiment. The factorial scheme was based on a seven-factor two-level plan which came down to eight runs (treatment combinations) per species, each spiked with 12 concentrations, resulting in plasma concentrations of 0.05, 0.10, 0.15, 0.25, 0.50, 1.0, 2.0, 4.0, 8.0, 16, 32 and 64 $\mu g\ ml^{-1}$ plus one blank sample for quality control purposes. A total number of 520 individual samples had to be investigated. The whole experiment was carried out within 5 weeks. The matrix calibration curves were established including the entire concentration range. All measurement values were taken into account (without eliminating potential outliers) for the calculation of the power curves and the critical concentrations using the lowest concentration (0.05 $\mu g\ ml^{-1}$) as the threshold level, i.e. the curve was not extrapolated. The overall as well as the matrix-specific calibration functions were calculated by means of weighted regression. The weights were determined based on a restricted maximum-likelihood approach for the determination of the concentration-dependent repeatability standard deviations.

The validation was carried out with underlying α- and β-error probabilities of 1% each for the calculation of the power curve and the critical concentrations CC_α and CC_β. The prediction interval was calculated with an underlying α-error probability of 5%. Standard curves were established in a targeted way according to the analyte concentration expected in the sample. The following standard curve levels were used in the validation experiment (Table 1).

The regression parameters are listed in Table 2.

4. Identification criteria

In accordance with Commission Decision 93/256/EEC [14] the analytes detected by means of HPLC–PDA were identified and confirmed by assessing

Table 3
Regression parameters of the overall calibration curves of the nine substances included in the validation experiment, calculated on the basis of the 40 calibration runs and 12 concentrations each.[a] The in-house repeatabilities and reproducibilities are given for three different concentrations: 0.1, 1.0 and 32 μg ml^{-1} [b]. The recoveries were calculated for each concentration. The lowest concentrations showed less recoveries than the higher concentrations

	Retention time (min)	Intercept $\hat{a}_0$ (area units)	Slope $\hat{b}_0$ (area, μg ml^{-1})	In-house repeatability[a] (%)	In-house reproducibility[a] (%)	CC_α (μg ml^{-1})	CC_β (μg ml^{-1})	Recovery[b] (%)
SA	8.9±1.2	0.226	10.5	30;30;30	63;41;41	0.184	3.60	39–76
OPB	16.29±0.035	−0.126	8.57	24;22;20	31;27;24	0.088	0.260	55–99
FLU	17.03±0.071	−0.058	21.4	9;9;9	11;10;10	0.042	0.057	72–111
CPF	19.70±0.035	−0.092	43.8	10;10;10	11;12;12	0.063	0.086	85–104
DC	20.89±0.035	−0.107	40.5	7;7;7	10;9;9	0.061	0.081	90–111
NFA	21.53±0.074	−0.325	86.2	8;8;8	10;10;10	0.060	0.078	91–111
PBZ	22.24±0.036	−0.659	42.8	18;18;18	24;23;23	0.086	0.184	63–99
MFAS	23.55±0.049	−0.048	32.5	8;8;8	14;10;9	0.076	0.111	94–110
VDP	27.30±0.046	−0.132	31.9	8;7;7	9;9;8	0.064	0.084	87–111

their retention time in connection with the spectral library tests. For this purpose a library of standard solutions of the respective analytes had been established beforehand, which was then compared to the analyte's spectrum in the sample. The absorption maxima in the spectrum of the analyte should be at the same wavelengths as those of the standard analyte within a margin determined by the resolution of the detection system. For diode array detection this is typically ±2 nm. The spectrum of the analyte above 220 nm should not be visually different from the spectrum of the standard analyte for those parts of the two spectra with a relative absorbency ≥10%. This criterion was met when the same maxima were present and the difference between the two spectra did not exceed 10% of the absorbency of the

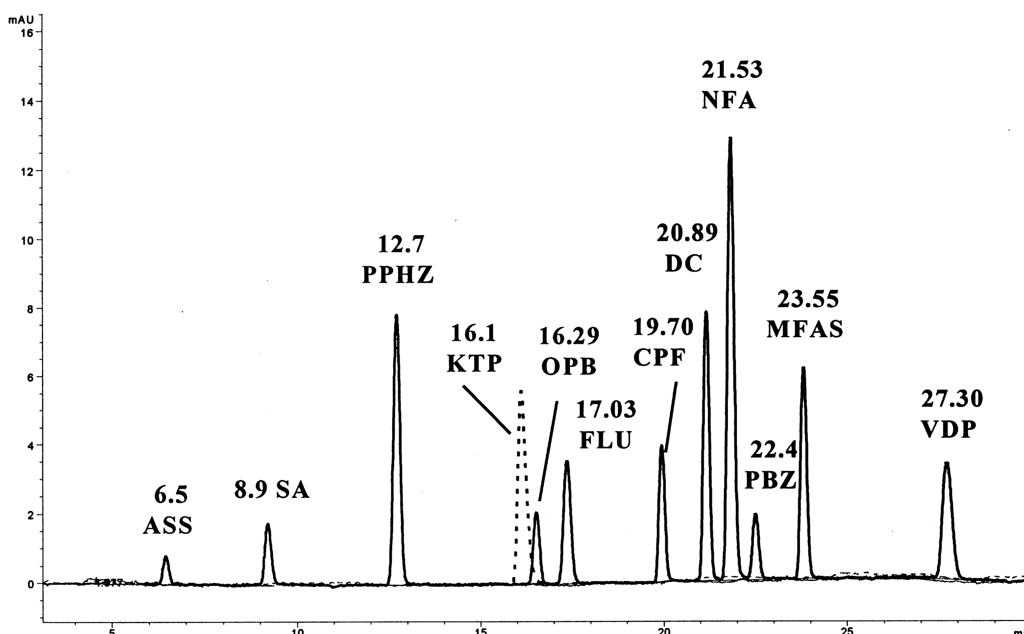


Fig. 3. Chromatogram of standard solutions of the validation mix (SA, PPHZ, OPB, FLU, CPF, DC, NFA, PBZ, MFAS, VDP), KTP and ASS (100 ng). KTP is not baseline separated from OPB. Therefore, it could not be included in the validation experiment. KTP and OPB can be distinguished by their UV spectra.

standard analyte at any point monitored. Co-chromatography in the LC step is mandatory for confirmatory purposes unless the method is used in combination with other methods. Commission Decision 93/256/EEC specifies certain conditions that have to be fulfilled in the case of co-chromatography.

5. Expression of results

The NSAID content was expressed in micrograms per millilitre using three significant places. The repeatability calculated during the validation process served as one part of the precision data. According to the validation results a recovery correction of prospective results will become necessary for SA, OPB and PBZ (Table 3). All other analytes investigated showed a recovery of about 100%. The calculation of contents was carried out using the area of the determined peaks from the UV chromatogram of the individual analytes. The content was calculated by linear regression of the standard curves according to the formulas for external standard calibration of the HP ChemStation software [15].

6. Results and discussion

The method presented was validated for SA, OPB, FLU, CPF, NFA, DC, PBZ, MFAS and VDP in the aforementioned matrices in the concentration range 0.05–64 ng ml^{-1}. The chromatograms of the investigated substances in standard solution are shown in Fig. 3.

Using the precision results (in-house repeatability and in-house reproducibility) and the critical concentrations (CC_α and CC_β) as performance criteria, it can be stated that the method is suitable for screening and confirmation of the aforementioned NSAIDs (except SA) in calf, bovine, ovine, porcine and equine plasma (see Table 3). It could be demonstrated that the method presented is applicable

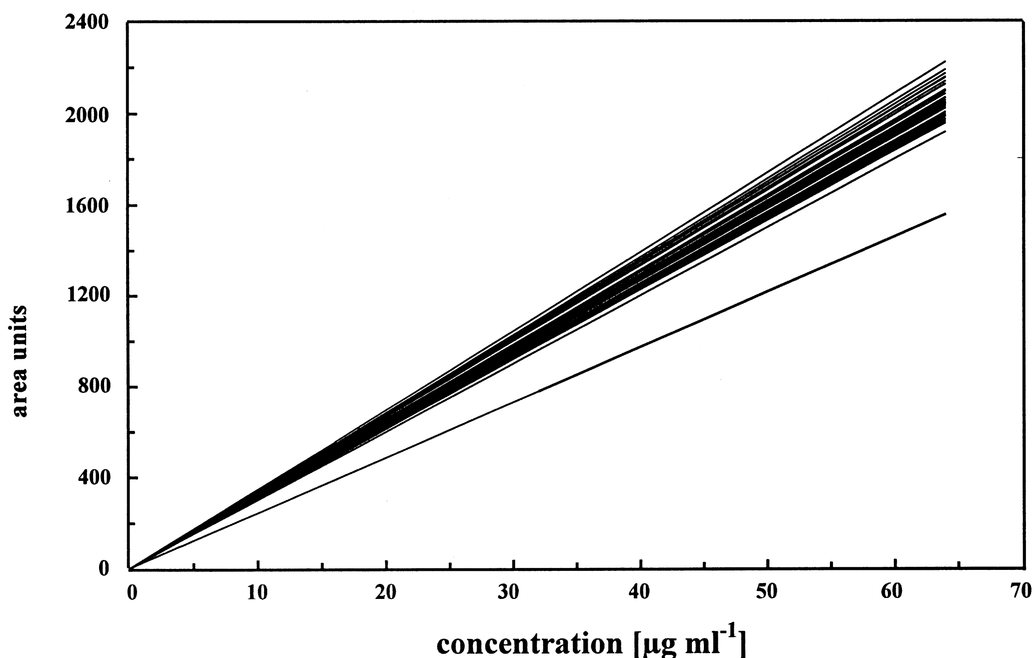


Fig. 4. Calibration curves of all 40 individual calibration runs of VDP.

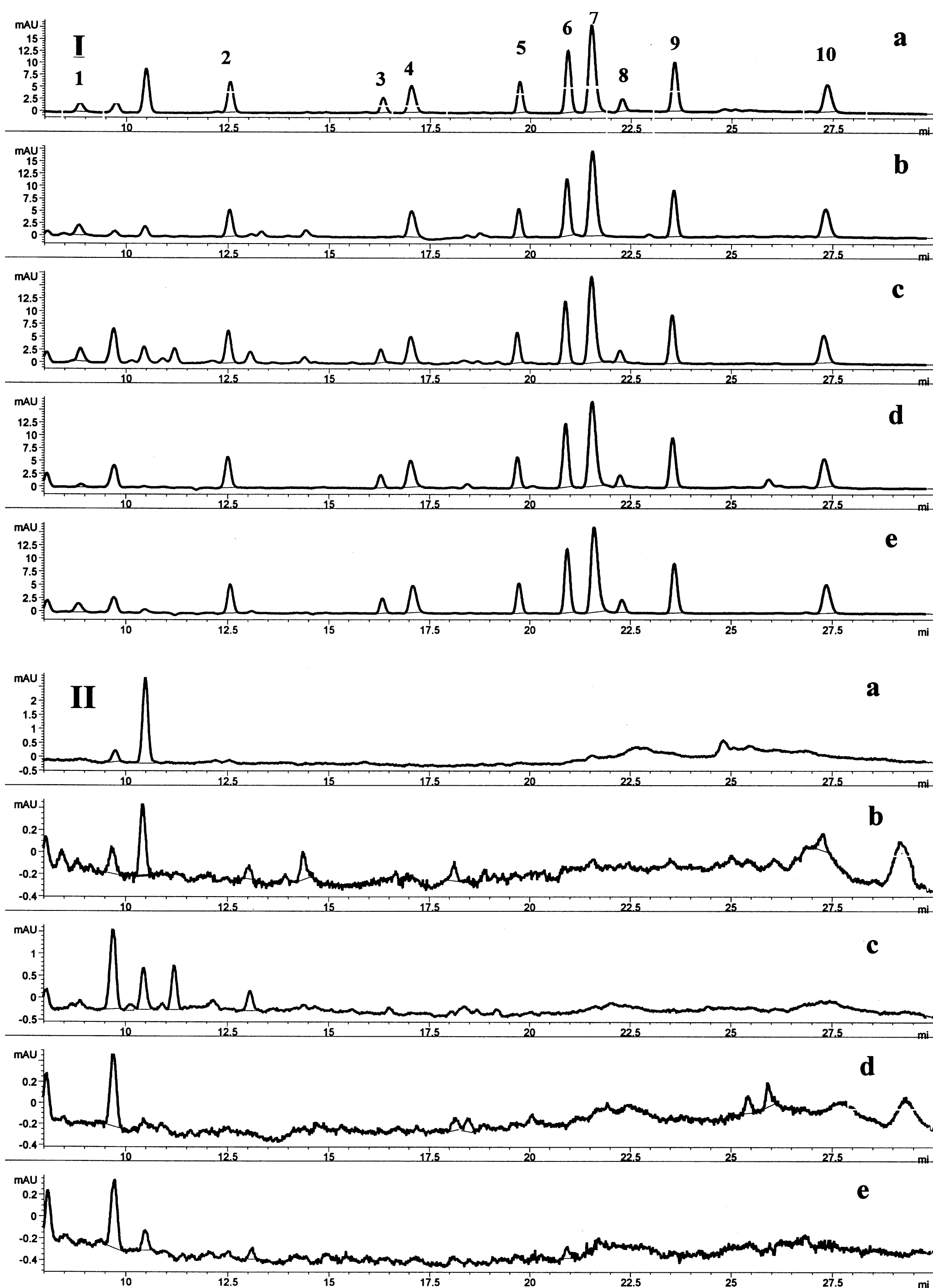


Fig. 5. Example chromatograms of all species at a plasma concentration of (I) 0.5 μg ml^{-1} and (II) blank samples. (a) Porcine, (b) ovine, (c) equine, (d) bovine and (e) calf plasma.

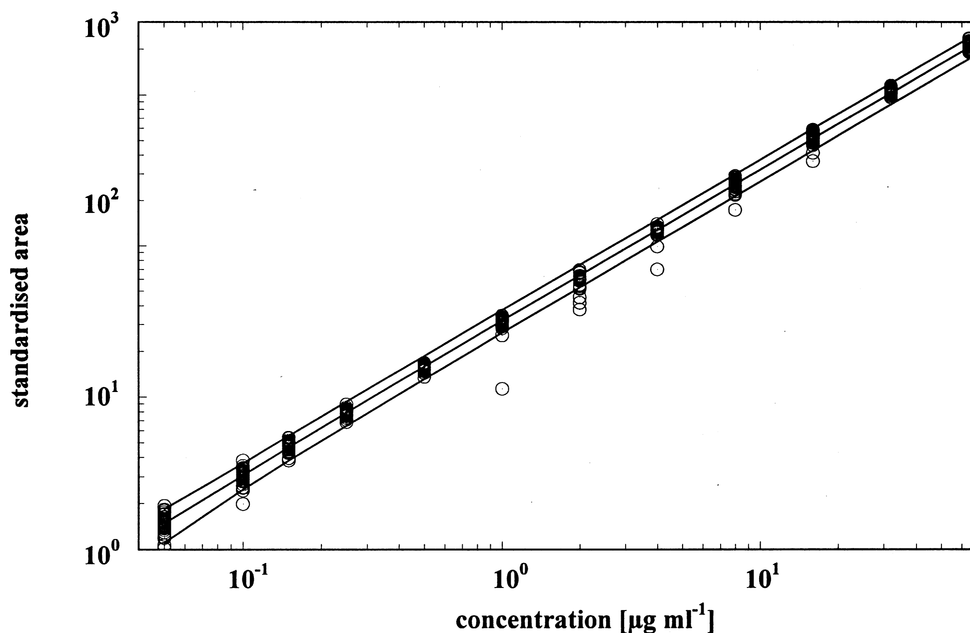


Fig. 6. Overall calibration curve with prediction interval, calculated on the basis of the 40 calibration runs for VDP.

to differently treated plasma samples, different feeding conditions for calves and bovines and that it is independent of both operational and personnel conditions. It produces linear matrix calibration curves in the validated concentration range independently of the mentioned potential influencing factors (Table 3, Fig. 4). The closeness of the scatter of the calibration curves also demonstrates the independence of the measurement results from time-induced variations (Fig. 4).

The validation experiment demonstrated that OPB and PBZ underwent partial to complete irreproducible degradation in approximately 1% of all samples, in a random manner. This phenomenon took place although ascorbic acid was applied as stabiliser (Fig. 5). Peaks of degradation products appeared at approx. 14 and 18 min retention time with spectra similar to those of OPB and PBZ. No structure elucidation of these peaks was performed. Therefore, the distribution of the measurement results for OPB and PBZ was distinctly larger than that of the other substances. SA underwent degradation and in some cases was affected by interfering matrix peaks (Fig. 5).

Thus the distribution of the quantitative and qualitative measurement results increased considerably and the identification criteria could not be fulfilled. Hence in the case of SA the method is only applicable for screening purposes and cannot be recommended for either quantification or confirmation of SA.

VDP was affected by interfering matrix components in some cases (Fig. 5), which, however, did not increase the distribution of the measurement results (Fig. 6). Nevertheless, the calculated CC_α and CC_β are still appropriate for control purposes (Table 3, Fig. 7).

As assumed according to the presumptions of the validation model [12], the in-house repeatability was independent of matrix influences as it did not decrease with increasing content levels. The influence of matrix effects on the in-house reproducibility became apparent when it decreased with increasing content levels (Table 3). In order to determine

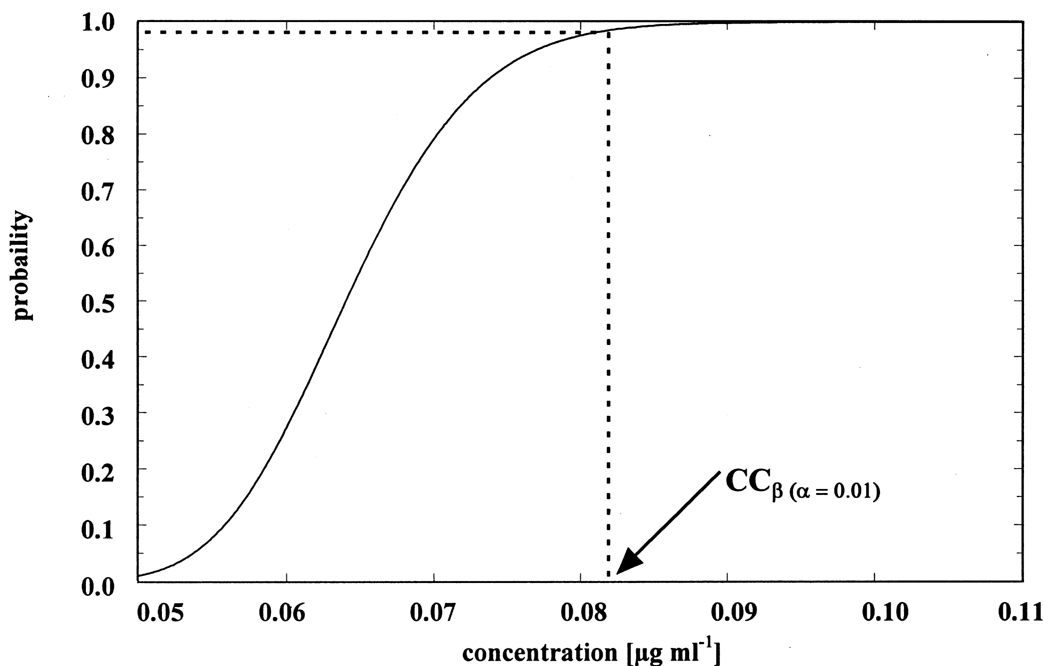


Fig. 7. Power curve of VDP, taking into account all sources of errors covered by the in-house validation concept.

possible existing dominating main effects, a variance analysis was performed for each of the three concentrations 0.1, 1.0 and 32 $\mu g\ ml^{-1}$ on the basis of the 40 calibration curves. The F-statistics for the main effect model were computed using the main effects species, sample volume, cooling, centrifugation, operator and storing. The following results were obtained for VDP as an example: $F=0.48$ for the concentration 0.1 $\mu g\ ml^{-1}$, $F=1.12$ for 1.0 $\mu g\ ml^{-1}$ and $F=1.20$ for 32 $\mu g\ ml^{-1}$. The overall results prove that there are no dominating main effects. The validation experiment showed that the method presented can be considered as successfully validated for OPB, FLU, CPF, DC, NFA, PBZ, MFAS and VDP. It could be demonstrated that the method is valid for a wide range of matrices and operational conditions. The critical concentrations CC_{α} and CC_{β} calculated for the different analytes (Table 3) are sufficiently low to ensure a sensible application of the presented method for residue control purposes.

7. Definitions

CC_{α}	The measured concentration from which it can be decided that an analyte is detected with the underlying error probability α for making a false-positive decision.
CC_{β}	The true concentration at which the probability of not detecting the analyte (false-negative rate) equals β.
In-house repeatability	Repeatability established by the in-house validation experiment considering the random measurement error.
In-house reproducibility	Reproducibility established by the in-house validation experiment considering matrix mismatch bias, run bias and random measurement error.

Acknowledgements

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