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Simultaneous determination of total and free drug plasma concentrations combined with batch-wise pH-adjustment for the free concentration determinations

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Abstract

Batch-wise pH-adjustment of plasma samples for free concentration determinations can be performed directly in the ultrafiltration devices using a CO_2 incubator. The pH of the samples could be adjusted to within a sufficiently narrow interval though the initial differences in pH were large. The possibilities for simultaneous determination of free and total concentrations were studied using NAD-299 as a model compound. By adding a known concentration of a ^{13}C-labelled isotope of the studied drug to the sample prior to ultrafiltration it was possible to calculate the total concentration from the ratio of the drug peak area to the isotope peak area while the free concentration was calculated from the drug peak area. Initial experiments showed good precision and accuracy as well as a good correlation with concentration data acquired in the conventional way.

Keywords: Free concentration; Ultrafiltration; Batch-wise pH-adjustment; NAD-299; μLC-MS/MS

1. Introduction

Drugs in the circulating blood are, more or less, bound to proteins in a reversible and kinetically rapid process. Only the unbound drug is pharmacologically active, therefore, the pharmacodynamics of a drug is thought to be better related to the free concentration and consequently free drug determinations have become a necessity in drug development.

A vast number of techniques have been used to study drug-protein binding, e.g. equilibrium dialysis, microdialysis, variants of size exclusion chromatography, ultracentrifugation and ultrafiltration [1,2]. For routine free drug determinations, ultrafiltration combined with chromatography has become the most used method as it is relatively fast and simple. Many commercially available filter systems with different molecular weight cut-offs can be found. Two drawbacks with ultrafiltration are frequently mentioned in the literature, problems with adsorption to the polymeric filters and plastic utensils and that ul-

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trafiltration concentrates the proteins on the retentate side and thus the binding equilibrium will change and the result will be dependant of the filtrate volume [2,3]. The adsorption problem must be checked for each substance under study while the disturbed equilibrium problem is probably a myth. Regarding the system as an equilibrium distribution of drug between two phases it is clear that taking away an equilibrated portion of any of the phases does not change the equilibrium in the system. This has been shown both in theory and in practice [4–6]. A more serious problem is the possibility to leakage of proteins through the filter, which in case of a highly bound drug can give large errors.

Many drugs contain amine functions with pK_a 7–8. These drugs often show a strongly pH dependent binding and it is therefore necessary to make the determinations at the physiologically correct pH value, 7.4. The pH in plasma is regulated by dissolved carbon dioxide. After thawing of a frozen plasma sample the pH is usually around eight and occasionally as high as nine, depending on age and storage conditions. The capacity limiting step in ultrafiltration methods is the individual treatment of each sample that is needed to restore the pH, by CO_2 purging or by other means. A less labour intensive procedure would be most appreciated.

Normally, the free concentration is determined as a complement to the total concentration, i.e. free and total concentrations are determined at separate occasions. Naturally it would be attractive to determine both concentrations simultaneously. Using high-performance frontal analysis (HPFA) it has been demonstrated that in certain cases both concentrations can be determined [7].

The possibility to simultaneously determine both concentrations by adding a ^{13}C-labelled isotope of the studied drug prior to ultrafiltration has, to our knowledge, not been recognized earlier.

This paper describes the application of batch-wise pH adjustment using a CO_2 incubator combined with simultaneous determination of free and total concentrations after addition of a ^{13}C-labelled isotope to the sample. NAD-299, a drug under clinical development, was used as model compound.

2. Experimental

2.1. Chemicals

NAD-299 [(R)-3-(*N*,*N*-dicyclobutylamino)-8-fluoro-3,4-dihydro-2*H*-1-benzopyran-5-carboxamide × (*R*,*R*)-tartrate monohydrate], the ^{13}C-labelled isotope of NAD-299 [(*R*)-3-(*N*,*N*-dicyclobutylamino)-(2,3,4-$^{13}C_3$)-8-fluoro-3,4-dihydro-2*H*-1-benzopyran-5-carboxamide × (*R*,*R*)- tartrate monohydrate] and the internal standard, NAE-111 [(*R*)-3-(*N*-isopropyl-*N*-propylamino)-3,4-dihydro-2*H*-1-benzopyran-5-carboxamide], were all synthesized at the Chemistry Department, Discovery, AstraZeneca R&D Södertälje, Sweden. The pK_a-value of NAD-299 is 7.4. The ^{13}C-labelled isotope was found to contain less than 0.1% of NAD-299. Injection of the same nominal concentration of NAD-299 and the labelled isotope gave, within 2%, the same mass spectrometric response.

2.2. Procedure for batch-wise pH adjustment experiments

Plasma samples of 1 ml were transferred to a standard 96-deep well plate (2 ml, quadratic wells). To get a large variation of initial pH, the plasma samples were a mixture of fresh samples, recently taken from healthy volunteers (initial pH ~ 7.8), and samples from old clinical studies (initial pH ~ 8.5). All samples were from different subjects. The 96-well plate was attached to a shaker (IKA Schüttler MTS 4, Janke & Kunkel, Staufen, Germany) inside a CO_2 incubator (HeraCell, Heraeus Instruments, Hanau, Germany) with adjustable temperature and percentage of CO_2. The pH-adjustment scheme was divided in two periods. The settings in the first period, 45 min long, were 37°C and 12% CO_2 and a shaking speed of 1100 rpm. In the second period, 30 min long, the CO_2 admixing was adjusted to 7%. After

that period the plate was sealed with a sealing tape and removed from the incubator.

The pH of each sample was measured before and immediately after the experiment using a Beckman Φ50 pH-meter (Beckman Instruments, Fullerton, CA, USA) equipped with a Beckman 39535 electrode.

2.3. Procedure for ultrafiltration experiments

Plasma samples of 1000 μl were transferred to Centricon YM-30 ultrafiltration devices (Millipore Corp., Bedford, MA, USA). The ^{13}C-labelled NAD-299 was added, 20 μl of a 5 μmol/l solution in blank plasma, thus giving a total concentration of 98 nmol/l. The ultrafiltration devices were placed in a tube rack and attached to the shaker in the incubator. The pH adjustments were made as above, 45 min at 12% CO_2 and 30 min at 7% CO_2. After this pH adjustment the samples were capped and immediately transferred to a centrifuge (Sorvall RT6000B, DuPont, Newtown, CT, USA), pH was checked in a selection of the samples and found to be within the accepted range. The ultrafiltration devices were centrifuged at 4000 *g* for 10 min and at 37°C, thereafter 200 μl ultrafiltrate from each collection tube was transferred to the autosampler vials.

2.4. Chromatography

The chromatographic system contained a Famos autosampler with two extra valves (Spark, Emmen, The Netherlands), two LC-10AD pumps (Shimadzu, Kyoto, Japan), one for analyte transfer and trapping and one for washing, and a Rheos 4000 pump (Flux Instruments, Karlskoga, Sweden) for delivering mobile phase to the analytical column. Detection was made using a Finnigan TSQ 7000 triple quadrupole mass spectrometer with electrospray interface (ThermoQuest, Riviera Beach, Florida, USA).

Without further treatment, 50 μl of the ultrafiltrate was transferred by 10 mM ammonium carbonate buffer, pH 7, from the autosampler to a micro trap column (0.5 × 5 mm, 5 μm Grom-Sil 120 ODS-5 ST, Grom Analytik, Herrenberg, Germany), flow rate 150 μl/min. The trapped analytes were eluted in the forward flush mode with the mobile phase (50% acetonitrile in 10 mM ammonium carbonate buffer, pH 7) of the analytical column on to the analytical micro column (0.8 × 100 mm, 3 μm Grom-Sil 120 ODS-5 ST), flow rate 40 μl/min. The system was washed by pumping through 50% acetonitrile in 10 mM ammonium acetate buffer, pH 3. After separation the analytes were detected by electrospray MS/MS, m/z 319 → 194 for NAD-299, m/z 322 → 197 for the ^{13}C-isotope and m/z 277 → 176 for NAE-111. The limit of detection, injected amount giving a peak 3 × noise, was found to be 200 attomoles.

To compensate for variations in MS/MS response during the run, the ultrafiltrate was injected together with 20 μl of a 4 nmol/l internal standard (NAE-111) solution. This was done automatically using the autosampler.

2.5. Calculations

The total concentrations of NAD-299, $C_{\mathrm{tot(drug)}}$, were calculated from:

$$C_{\mathrm{tot(drug)}} = C_{\mathrm{tot(Iso)}} \times \mathrm{Area}_{\mathrm{drug}}/\mathrm{Area}_{\mathrm{Iso}},$$

where $C_{\mathrm{tot(Iso)}}$ is the added concentration of ^{13}C-labelled isotope and $\mathrm{Area}_{\mathrm{drug}}$ and $\mathrm{Area}_{\mathrm{Iso}}$ are the areas for the drug and isotope peak, respectively.

The free concentrations, $C_{\mathrm{free(drug)}}$, were calculated using the internal standard (IS) added prior to injection from the following equation:

$$C_{\mathrm{free(drug)}} = C_{\mathrm{drug(calib)}} \times \mathrm{Area}_{\mathrm{drug}} \times \mathrm{Area}_{\mathrm{IS(calib)}}/(\mathrm{Area}_{\mathrm{drug(calib)}} \times \mathrm{Area}_{\mathrm{IS}}),$$

where $\mathrm{Area}_{\mathrm{drug}}$ and $\mathrm{Area}_{\mathrm{IS}}$ are the areas for the drug and internal standard peak in the sample and $\mathrm{Area}_{\mathrm{drug(calib)}}$ and $\mathrm{Area}_{\mathrm{IS(calib)}}$ are the average from six injections of a 4 nM ($C_{\mathrm{drug(calib)}}$) standard solution. The free fraction, ff, is calculated from:

$$\mathrm{ff}_{\mathrm{drug}} = C_{\mathrm{free(drug)}}/C_{\mathrm{tot(drug)}}.$$

2.6. Performance

Linearity was tested, for both total and free concentrations, from a regression plot comprising eight samples with total concentrations ranging from 10 to 300 nmol/l, prepared by adding NAD-

299 to blank plasma. In the same experiment also six samples with 25 nmol/l and six samples with 250 nmol/l were run to give an idea about the within-day accuracy and precision. The same blank plasma was used in all samples and all twenty samples were ultrafiltered as described above. The total concentrations were back-calculated from the slope of the regression plot while all free concentrations were calculated as mentioned above.

Twenty samples from a clinical study were also analysed and the resulting concentrations were compared to old data. The total concentrations in these samples were earlier determined using a LC-MS/MS method with liquid–liquid extraction as sample work-up. The free concentrations were determined earlier, not in the same samples but in samples taken at the same occasion, by using conventional individual CO_2 purging and ultrafiltration followed by determination using the same μLC-MS/MS system as in this study. These total and free concentrations were both determined 20 months earlier and the samples were 2 years old at the time of the experiments described in this article.

3. Results and discussion

3.1. Batch-wise pH adjustment

As the storage time and conditions varied for the plasma samples used in this experiment, the variation in initial pH of these samples was much larger than what is observed in normal cases. The initial pH in the fresh samples ranged from 7.80 to 8.31 while the range in the old samples was from pH 8.36 to 9.04. The goal was to adjust the pH of all samples to between 7.30 and 7.45 and to have an end-point pH independent of the initial pH. It proved beneficial, in a time perspective, to start the pH adjustment using a rather high admixing of CO_2, 12%, and then go down to 7% CO_2 for a second period. The length of each period must probably be optimized for each instrumental set-up but in our case we found that 45 min at 12% CO_2 and then 30 min at 7% CO_2 gave the best results. If the first period was shortened, the endpoint pH tended to be too high. If the second period was shortened the variance in the endpoint pH increased. Fig. 1 shows that the endpoint pH in the samples after the batch-wise pH adjustment was within the accepted limits and that the endpoint pH was independent of the initial pH.

3.2. Simultaneous determination of free and total concentrations

Ultrafiltration has become the most popular way of making routine free concentration determinations. Plasma is, usually after temperature adjustment to 37°C and pH adjustment to 7.4, filtered through a polymeric membrane with a narrow molecular weight cut-off, normally 30 kD. Filtering devices are available from several manufacturers. After the ultrafiltration, protein and bound drug are left in the retentate while the filtrate consists of free drug in plasma water. Obviously, determining the drug concentration in the filtrate gives the free concentration, C_{free}. A ^{13}C-labelled isotope of the studied drug is assumed to have close to identical protein binding, retention and MS-response but has a different mass, making it detectable by MS although it co-elutes with the drug peak. Adding this isotope to the sample prior to ultrafiltration means that it will mimic the behaviour of the drug and give the

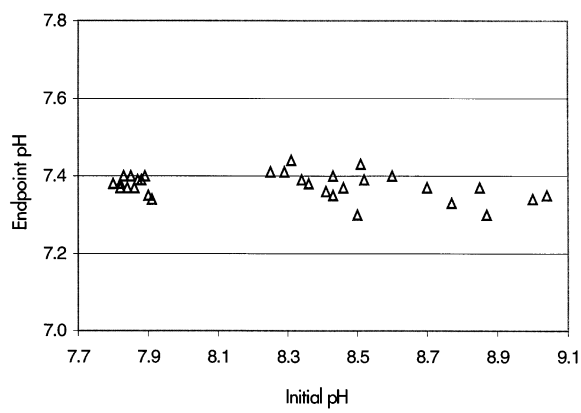

Fig. 1. Initial pH versus endpoint pH after batch-wise pH-adjustment. Data from two experiments, 16 samples in each experiment, are presented together.

Table 1
Within-day accuracy and precision.

Added total concentration	250 nM		25 nM	
	Free (nM)	Total (nM)	Free (nM)	Total (nM)
Average	2.0	242	0.19	24.9
SD	0.11	6.6	0.016	1.5
Rel SD	5.6	2.7	8.5	5.9
n	6	6	6	6

same free fraction (ff) in the filtrate. The free fractions are calculated from:

$$ff_{Iso} \times C_{tot(iso)} = C_{free(Iso)},$$

$$ff_{drug} \times C_{tot(drug)} = C_{free(drug)}$$

The free fraction of the drug used as model compound in this study is constant up to at least 1 μM. As long as the sum of drug and isotope, irrespective of differences in concentration, is below that, then

$$ff_{Iso} = ff_{drug}.$$

The chromatographic peak area is, as always, proportional to the concentration, so if the concentration of the added isotope is known the total drug concentration can then be calculated from:

$$C_{tot(drug)} = C_{tot(Iso)} \times Area_{drug}/Area_{Iso}.$$

Thus the free concentration is calculated from the drug peak area and the total concentration is calculated from the ratio of the same drug peak area to the isotope peak area.

When the determinations are performed in a concentration range where the binding is concentration dependent the addition of the ^{13}C-isotope will shift the free fraction. Provided that the addition of isotope is kept small, $< 5–10\%$ of the total drug concentration, the change in free fraction will however, be insignificant and thus good results for the free concentration can be obtained also in the concentration dependent range. It should be pointed out that the total concentration results are always correct.

3.3. Chromatography

The model compound, NAD-299, chosen for demonstrating the applicability of the described improvements put high demands on the sensitivity of the analytical method with its combination of low drug levels and high protein binding. The patient plasma samples used in this study were taken from a phase I study with NAD-299 levels roughly between 10 and 300 nmol/l and with free fractions of about 1%. The lowest concentration in the regression plot discussed below corresponded to an injected amount of 4 fmol. The demanded sensitivity was accomplished by a combination of large injection volumes, a μLC system where the injected ultrafiltrate was concentrated on a trap column, and MS/MS detection.

3.4. Performance

A regression plot with eight points at spiked concentrations of 10–300 nmol/l was linear when calculated from the total concentrations, R^2 was 0.99996. Also the plot for the free concentrations showed good linearity, $R^2 = 0.997$.

The within-day accuracy and precision is presented in Table 1. Generally the accuracy was very good. The precision for the free concentration determinations was acceptable and rather typical

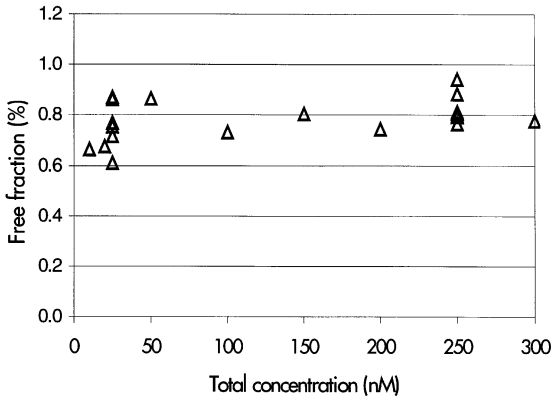

Fig. 2. Free fractions determined in plasma spiked with different concentrations of NAD-299.

for ultrafiltration methods. The precision was much better for the total determinations, as expected when considering the use of the ^{13}C-labelled isotope as internal standard. Also the free fraction was calculated for all 20 samples in this set, to see if the free fraction was constant in the chosen concentration interval. That was indeed the fact, as can be seen in Fig. 2. The average free fraction was 0.78% with a relative standard deviation of 10.3% ($n = 20$). The comparison with old total concentration results demonstrates that a good agreement was found (Fig. 3), although there was a tendency to slightly lower values for

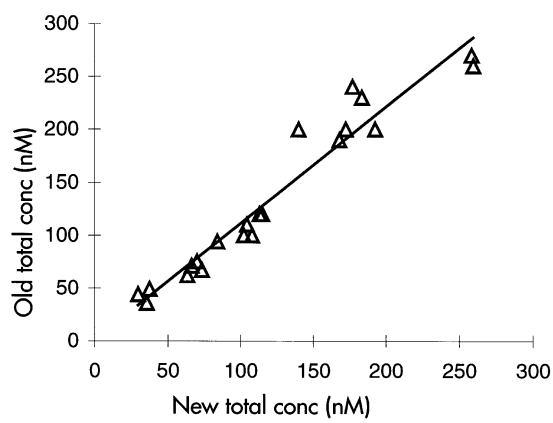

Fig. 3. Comparison between total concentrations determined according to the described procedure and total concentrations determined earlier using a liquid-liquid extraction LC-MS/MS method.

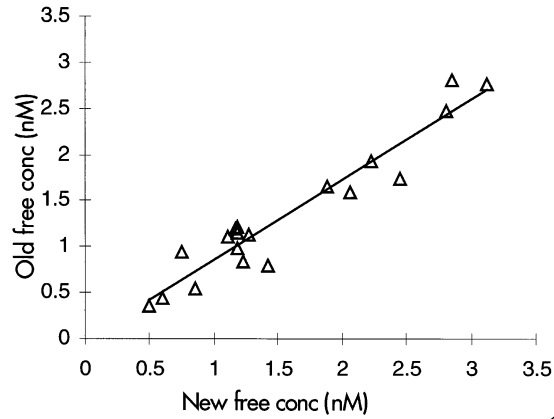

Fig. 4. Comparison between free concentrations determined according to the described procedure and free concentrations determined earlier using a conventional pH-adjustment method.

the newly determined concentrations (slope = 1.09, $R^2 = 0.94$). For the free concentration determinations there was still a rather good agreement (Fig. 4) but instead a tendency for higher new values (slope = 0.87, $R^2 = 0.92$).

3.5. Remark

When the idea of simultaneous determination of free and total concentrations came up, automation via the application of 96-well plate technology and robotic liquid handling was the obvious goal. Presently there are no standard format 96-well plates for ultrafiltration available. We made the pH-adjustment experiments in 96-well plates with excellent results but ultrafiltration had to be performed in conventional devices. A standard format 96-well ultrafiltration plate will make the presented application even more attractive.

3.6. Conclusion

The initial experiments described here show that both batch-wise pH-adjustment and simultaneous determination of free and total concentrations can be used to drastically reduce time and money spent on free concentration determinations.

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