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Practice of solid-phase extraction and protein precipitation in the 96-well format combined with high-performance liquid chromatography–ultraviolet detection for the analysis of drugs in plasma and brain

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Abstract

C_{18} Empore 96-well extraction disc plates have been employed for the analysis of three drugs with different polarities in plasma in conjunction with HPLC–UV, rufinamide, ICL670 and an anticonvulsant agent (AA1) in an early stage of development. With the most polar compound (AA1), ion-pair extraction at pH 12 was applied. The method developed for the assay of AA1 in plasma was applied to its determination in brain using an Oasis HLB plate following homogenisation in a pH 7.4 buffer and protein precipitation with $NaOH–ZnSO_4$, thereby saving time for method development. Protein precipitation in the 96-well format with filtration of the precipitate was applied to the determination of ICL670, a highly protein-bound compound (>99.5%), with a good recovery (78%). Reversed-phase chromatography was applied using a short 5 cm column packed with 3 μm particles for the determination of ICL670 and AA1 and two parallel columns (15 cm long) for the determination of rufinamide. The methods were used routinely, one plate per analysis day being processed, resulting in increase in sample throughput and saving in solvents.

Keywords: Protein precipitation; Rufinamide; ICL670

1. Introduction

The need for fast and efficient bioanalytical procedures within the pharmaceutical sector is rapidly growing. Sample preparation is often the bottleneck step, so that tremendous efforts are being made to speed up this step. There is a recent trend for batch sample preparation in the 96-well format as a tool for high sample throughput sample preparation, 96 samples being processed simultaneously. The feasibility to use 96-well plates has been successfully demonstrated for different sample preparation techniques. Liquid–liquid extraction (LLE) has been semi-automated using 96-well plates instead of individual tubes, the volumes of solvent being smaller [1,2]. Solid-phase extraction (SPE) is more frequently being applied using 96-well SPE extraction plates instead of individual columns or cartridges [3–5]. Protein precipitation has also recently been adapted to the 96-well format for the determination of a weakly protein-bound compound in plasma, the centrifugation step being replaced by a filtration step [6].

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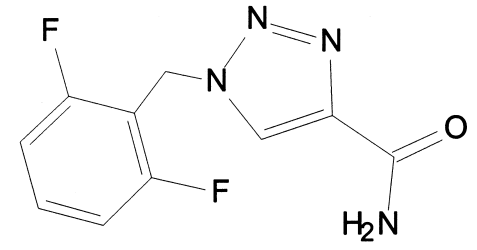
SPE is becoming more and more popular as a result of the development of a large choice of sorbents for trapping analytes with a wide range of polarities [7] and because it provides an opportunity for automated sample preparation. Extraction plates with sorbent bed amounts smaller than in traditional cartridges (5–15 mg per well vs. 50–100 mg per cartridge) have been introduced, allowing for smaller elution volumes. New selective sorbents have recently been made available commercially in the 96-well format. Copolymeric sorbents called hydrophilic–lipophilic balanced (HLB) sorbents are now provided in plates [8,9]. Compared to reversed-phase silica phases, they have a higher binding capacity, are water-wettable, are stable over an extended pH range 0–14, and have predictable mechanisms of interaction with no secondary interactions with residual silanols. Mixed-mode sorbents contain reversed-phase alkyl chains and ion exchangers bonded on the same solid [10]. They allow for a high selectivity since they involve both hydrophobic interactions and ion-exchange and may be a compromise when the high ionic strength of the plasma precludes pure ion-exchange retention. Dual Zone sorbents have been recently proposed in the 96-well format. They combine size exclusion of high-molecular-mass analytes and retention of small analytes at the inner hydrophobic pore surface. The outer silica surface allows for hydrophilic interactions only. Another advance in the area of SPE in the 96-well format is the availability of particle-loaded membranes or membrane-extraction discs instead of conventional packings [11–15]. The Empore membrane consists of bonded silica particles immobilized within an inert matrix of polytetrafluoroethylene (90% particles). Due to a dense and uniform medium, the channelling is minimised and the sorbent bed amount is reduced compared to conventional packings while a high capacity is maintained. Since the membrane in each well is cut from the same sheet of material, uniform flow from well to well is expected. In addition, the Empore SPE extraction plates are equipped with a patented prefilter composed of polypropylene microfibres of graded densities, the coarsest one on top, and the finest at the bottom. This prefilter has been experimentally determined to retain 98% of all particles larger than 10 μm in size and 50% of particles as small as 2 μm. The eluate obtained is consequently expected to be free from particles which can be responsible for column clogging as this may occur in conventional SPE.

Sample preparation in the 96-well format is mainly used in conjunction with liquid chromatography–tandem mass spectrometry (LC–MS–MS) because this technique allows short analysis times due to its high specificity. A labelled internal standard with the same structure and retention time as the compound to be determined can be used, which further decreases the analysis time. However, when a high sensitivity is not required, optical detection is still used because it is less expensive than MS–MS detection and requires less expertise and less intervention. When UV detection is applied, sample preparation in the 96-well format can also enhance sample throughput provided the chromatographic step is adapted in order to decrease the run time. Some examples are described in this paper. The data reported are based on three Novartis compounds under development, rufinamide, ICL670 and AA1. Rufinamide and AA1 are anticonvulsant compounds, and ICL670 is an iron chelator. Bioanalytical methods for their determination in plasma have been developed. The methods are based on SPE in the 96-well format, LC and UV detection. An analog of each compound is used as internal standard. For rufinamide, a method previously reported [16] based on SPE with individual cartridges and conventional chromatography with a 15 cm long column packed with 5 μm particles was modified. For ICL670, an even more rapid sample preparation procedure involving protein precipitation and filtration in the 96-well format was also developed. For AA1, the feasibility to perform determinations in the brain in the 96-well format using the SPE method developed for the determination in plasma was investigated.

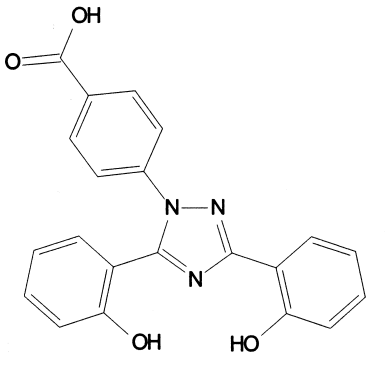
2. Experimental

2.1. Solvents and chemicals

Rufinamide, ICL670 and AA1 were synthesised by Novartis Pharma, Basle, Switzerland. The chemical structures of rufinamide and ICL670 are exhibited in Fig. 1. An analog of each compound was



RUFINAMIDE



ICL670

Fig. 1. Chemical structures of rufinamide and ICL670.

used as internal standard (I.S.). Analytical-grade methanol and acetonitrile were obtained from Carlo-Erba (Nanterre, France). Ethanol absolute was obtained from Prolabo (Fontenay sous Bois, France). Orthophosphoric acid 85%, Titrisol pH 5, 7 and 12, and Titrisol 0.1 and 0.5 *M* NaOH were obtained from Merck (Nogent sur Marne, France). $ZnSO_4$ and hexylamine were obtained from Sigma–Aldrich (St. Quentin Fallavier, France). Phosphate-buffered saline (PBS) solution (pH 7.4) from Amresco was supplied by Interchim (Montluçon, France). Water was deionized, filtered and purified on a Milli-Q reagent-grade water system from Millipore (St. Quentin en Yvelines, France).

Human plasma was obtained from Etablissement Français du Sang where blood was collected from volunteers in tubes containing citrate–phosphate–dextrose. After centrifugation, the plasma was transferred and stored at −20°C.

2.2. Standard solutions

Primary stock solutions were prepared by dissolving rufinamide in acetonitrile, ICL670 in ethanol and AA1 in 0.1 *M* NaOH. Appropriate dilutions of the stock solutions with water–acetonitrile (70:30, v/v) for rufinamide, pH 7 buffer for ICL670 and AA1, were then made in order to prepare the spiking solutions. Solutions were similarly prepared for the internal standards. Two series of stock solutions were prepared from different weighings to spike the calibration and quality control plasma samples. All the solutions were prepared in glass flasks and stored at 4°C .

2.3. High-performance liquid chromatography (HPLC) apparatus and chromatography

The HPLC system consisted of a Model 305 pump from Gilson (Villiers-le-Bel, France), an autosampler ASPEC from Gilson, a Model UV-975 Jasco detector from Merck monitoring at a wavelength of 230 nm (rufinamide), 295 nm (ICL670) or 316 nm (AA1). For the determination of rufinamide, a Model LC-10 AD Shimadzu pump supplied by Touzart et Matignon (Courtaboeuf, France) and a Must 2177 valve switching system (Spark, Emmen, The Netherlands) were also used. A chromatography workstation, Model X-Chrom from LabSystems (Issy-Les-Moulineaux, France), was used to perform data acquisition.

The chromatographic separations were performed as follows:

Rufinamide: two columns were placed in parallel. An injection was performed every 15 min onto the columns each one in its turn. Each column was connected to the detector during the first 15 min after the injection and the effluent was directed to waste during the next 15 min according to the scheme shown in Fig. 2. Supelcosil LC-18 columns, 150 mm×4.6 mm, 5 μm particle size, supplied by Sigma–Aldrich, and Supelguard LC-18 pre-columns (from Sigma–Aldrich), 20 mm×4 mm, 5 μm particle size, were used. The chromatography was performed at ambient temperature. The mobile phase, acetonitrile–methanol–0.02 *M* KH_2PO_4 (18:8:74, v/v), was delivered at a flow-rate of 1.2 ml/min from 0 to 15 min in the forward flush mode

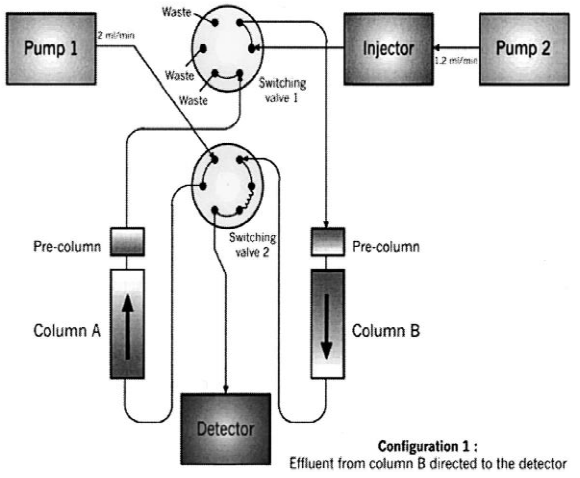


Fig. 2. Column switching configuration. With the represented configuration of the automated switching valve, the effluent from column B is directed to the detector while column A is back-flushed. When the valve is switched, the effluent from column A is directed to the detector while column B is back-flushed.

and of 2 ml/min from 15 to 30 min in the backward flush mode.

ICL670 and AA1: an Alltima C_{18} column, 50 mm×4.6 mm, 3 μm particle size, supplied by Alltech (Templemare, France), was used for the determination of both compounds. The following pre-columns were used, KS 11/4 Nucleosil 100-5 C_{18} (from Macherey-Nagel, Hoerdt, France), 11 mm×4 mm, 5 μm particle size, for the determination of ICL670 (SPE) and AA1, and LiChrospher RP18, 4 mm×4 mm, 5 μm particle size, supplied by Merck, for the determination of ICL670 (precipitation of proteins). The chromatography was performed at 50°C. The mobile phase, methanol–ethanol–0.005 *M* H_3PO_4 (22:38:40, v/v/v), was delivered at a flow-rate of 1.3 ml/min for the determination of ICL670. Acetonitrile–0.005 *M* H_3PO_4 (15:85, v/v) was delivered at a flow-rate of 1 ml/min for the determination of AA1.

2.4. Sample preparation

2.4.1. Extraction from plasma

All liquid transfers were done manually using Biohit Proline electronic pipettors (Bonnelles, France). Twelve-channel pipettors were used in the multi-dispensing mode whenever possible. The plasma was thawed and 50 μl (ICL670), 100 μl (rufinamide) or 200 μl (AA1) of centrifuged plasma was transferred to the appropriate well of a deepwell source plate. An equal volume of water (rufinamide), 1 *M* H_3PO_4 (ICL670), or 0.01 *M* hexylamine (AA1) was then added. Sodium hydroxide (0.1 *M*, 120 μl) was further added for the determination of AA1. After addition of an aliquot of the internal standard solution (and of the solution of the compound to determine for calibrators and quality control samples), the plate was vortex-mixed for a few seconds. A 3M Empore C_{18} disc plate (supplied by Varian, Les Ulis, France) containing 14 mg sorbent per well was placed on top of a 3M vacuum manifold. The extraction was then applied according to the procedures described in Table 1. The vacuum was

Table 1
SPE procedures for the determination of rufinamide, ICL670 and AA1 in plasma

Step	Fluid
Rufinamide	
Sorbent,	100 μl acetonitrile,
conditioning	100 μl water
Loading	210 μl diluted plasma
Washing	25 μl 0.02 *M* K_2HPO_4
	50 μl water–acetonitrile (90:10)
Elution	40 μl×2 acetonitrile
Dilution	300 μl water
Injection	200 μl
ICL670	
Sorbent,	200 μl acetonitrile,
conditioning	200 μl 0.01 *M* H_3PO_4
Loading	150 μl diluted plasma
Washing	50 μl 0.02 *M* K_2HPO_4
	50 μl water
	50 μl 0.01 *M* H_3PO_4–acetonitrile (70:30)
Elution	50 μl×2 acetonitrile
Dilution	150 μl 0.01 *M* H_3PO_4
Injection	100 μl
AA1	
Sorbent,	200 μl methanol,
conditioning	200 μl 0.01 *M* hexylamine
Loading	500 μl diluted plasma
Washing	100 μl buffer, pH 12
	50 μl buffer, pH 12–methanol (70:30)
	50 μl buffer, pH 5
Elution	100 μl×2 methanol
Dilution	150 μl 0.005 *M* H_3PO_4
Injection	100 μl

applied to aspirate the liquids after each dispensing step till the wells were still wet for the conditioning and loading steps and all wells were dried for the other steps. A collection plate with 96 polypropylene tubes fit in the standard 9 mm centre-to-centre spacing was placed in the bottom of the vacuum manifold after the last washing step instead of the waste collection plate. Following the elution step, the extract was diluted, and the collection tubes were placed on the autosampler rack refrigerated at 4°C.

2.4.2. Plasma protein precipitation

A 100-μl volume of thawed plasma was transferred to a deepwell source plate and an aliquot of the internal standard solution (and of the appropriate ICL670 spiking solution for calibrators and quality control samples) was added. The plate was vortex-mixed for a few seconds. A 3M Empore filter plate PPT was placed on top of a 3M vacuum manifold. A 200-μl volume of water was dispensed above each filter of the filter plate, and a slight vacuum was applied. A collection plate with 96 polypropylene tubes was then placed in the bottom of the manifold. A 300-μl volume of acetonitrile and 100 μl spiked plasma were subsequently withdrawn, separated with an air gap, using an electronic single-channel pipettor working in the dilution mode. The liquids were then dispensed together above each filter, to the inner side of the well. Once all wells were filled, a gentle vacuum was applied until the collection tubes were filled and each well was dried. Then, the collection tubes were placed on the autosampler rack refrigerated at 4°C. Prior to injection, 200 μl of 1 *M* orthophosphoric acid was automatically added into each collection tube by the ASPEC system, and 200 μl was injected.

2.4.3. Extraction from brain

Brains were obtained from rats following administration of AA1. The brains were weighed and cut up, and 1 μl/mg PBS buffer, pH 7.4 was added. The mixture was homogenized with a Polytron homogenisator (Bioblock, Strasbourg, France). A 200-μl volume of the homogenate was transferred to a tube. After addition of an aliquot of the internal standard solution (and of the solution of AA1 for quality control samples), 200 μl 0.5 *M* NaOH–($ZnSO_4 \cdot 7H_2O$) (10%, v/w) was added. The tube was shaken on a vortex mixer for 1 min, and 200 μl of 0.01 *M* hexylamine and 120 μl of 0.1 *M* NaOH were added. The tube was vortex-mixed again and centrifuged for 5 min at 4500 rpm. Then, the extraction procedure described in Table 1 for the determination of AA1 in plasma was applied using an Oasis HLB 96-well extraction plate and a 3M vacuum manifold. The supernatant (550 μl) was loaded onto the plate instead of the diluted plasma. The calibration and quality control samples were prepared in plasma using 200 μl plasma instead of 200 μl homogenate.

3. Results and discussion

3.1. Extraction from plasma

C_{18} Empore disc 96-well extraction plates were used for the determination of the three compounds, rufinamide, ICL670 and AA1 in plasma. This sorbent gave satisfactory results in all cases when the ionisation of the compounds was minimised at the time of the loading step. Rufinamide is a neutral compound. Therefore, no pH adjustment was performed. The sorbent was conditioned with water and the plasma was diluted with water prior to extraction. ICL670 is more lipophilic ($\log D_{7.4\ \text{octanol–buffer}} = 0.8$) than rufinamide. It was extracted at acidic pH in order to suppress the ionisation of its acidic carboxylic function ($pK_a = 4.6$) and to convert the iron complex into the parent drug [17]. AA1 is a very polar ($\log D_{7.4\ \text{octanol–buffer}} = -4.6$) amphoteric compound with two acidic functions ($pK_a = 1.5$ and 4) and two basic functions ($pK_a = 6$ and 10.5). The extraction of AA1 was performed at pH 12 to suppress the ionization of its two basic functions, and an ion-pairing agent, hexylamine, was added to decrease the polarity of its acidic functions. AA1 was strongly absorbed with the loading conditions used. Three different washings were applied, the last one with pH 5 buffer, in order to start the desorption of AA1 strongly ionized at this pH around its isoelectric point. Pure methanol was found to be necessary for the elution. The recovery of the extraction was determined from the comparison of the UV responses obtained from spiked plasma samples and spiked blank plasma extracts. It was

68% for rufinamide, 99% for ICL670 and 58% for AA1 as a mean.

The extraction of AA1 was realized at a high pH where the silica is known to dissolve. However, only a slight increase in the column pressure was noticed over the course of an analytical run when using Empore disc C_{18} plates for the extraction, thereby suggesting that the dissolution of the small amount of silica per well (14 mg) at the high working pH was not sufficient to cause system failure. The extraction was also performed using an Oasis HLB 96-well extraction plate known to be stable up to pH 14. Prior conditioning of the sorbent with hexylamine was essential in order to avoid losses during the loading step as well for the copolymeric HLB plate as for the C_{18} Empore plate. The two sorbents resulted in similar selectivity while a slightly better recovery (about 60% instead of 50%) was obtained with the C_{18} sorbent. This might have been due to more difficulties to desorb AA1 from the highly retentive HLB sorbent.

Due to the small mass of sorbent in each well, solvent volumes as low as 80–100 μl for rufinamide and ICL670 and 200 μl for AA1 were sufficient to elute the compounds. This allowed for direct injection of the eluate after dilution with an aqueous solution, thereby avoiding evaporation and reconstitution which are time consuming and may yield problems due to thermal instability of the analyte. The solvent volumes used for conditioning and washing (25–200 μl) were also lower than those required for traditional SPE cartridges (around 1 ml), which is a significant advance in sample preparation miniaturisation.

ICL670 and AA1 were selectively detected at wavelengths around 300 nm where little endogenous compounds absorb. A short 5 cm analytical column packed with 3 μm particles was used for the determination of both compounds. The chromatographic run time was 6–7 min, thereby allowing a high sample throughput compatible with the use of 96-well plates. Chromatograms are shown in Figs. 3 and 4. Rufinamide absorbs at low wavelengths only, and the run time could not be decreased by using a short column because of numerous peaks arising from endogenous components in the blank. Moreover, rufinamide is commonly administered with concomitant anti-epileptic drugs which are strongly

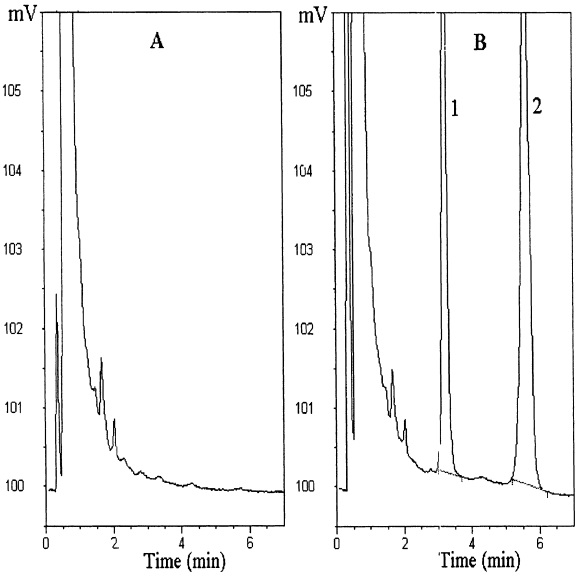


Fig. 3. Representative chromatograms obtained following extraction of ICL670. Peak 1: ICL670, peak 2: I.S. (A) Drug-free plasma, (B) plasma spiked with ICL670 (5000 ng/ml) and I.S.

retained on the analytical column [16]. Concomitant carbamazepine and phenytoin are extracted with the described procedure and are eluted with retention times of 30–40 min. The use of two parallel columns permitted to increase sample throughput, the retained concomitant anti-epileptic agents being eluted in the backward flush mode from one column while rufinamide was determined on the other column. The chromatographic analysis time was then 15 min allowing for the injection of a plate a day. Chromatograms are exhibited in Fig. 5. The column was efficiently washed in the backward flush mode since there was no interference at all arising from a previous injection.

Calibration samples were prepared at five different concentrations in duplicate in the range 50–20 000 ng/ml for rufinamide, 100–50 000 ng/ml for ICL670, 25–5000 ng/ml for AA1. The calibration curves, represented by the plots of the peak area or height ratio parent compound/I.S. versus parent compound concentration in the calibration sample, were generated using weighted ($1/x^2$) linear regression. Linear calibration curves were obtained with generally a coefficient of correlation higher than 0.99. Six replicate quality control samples were

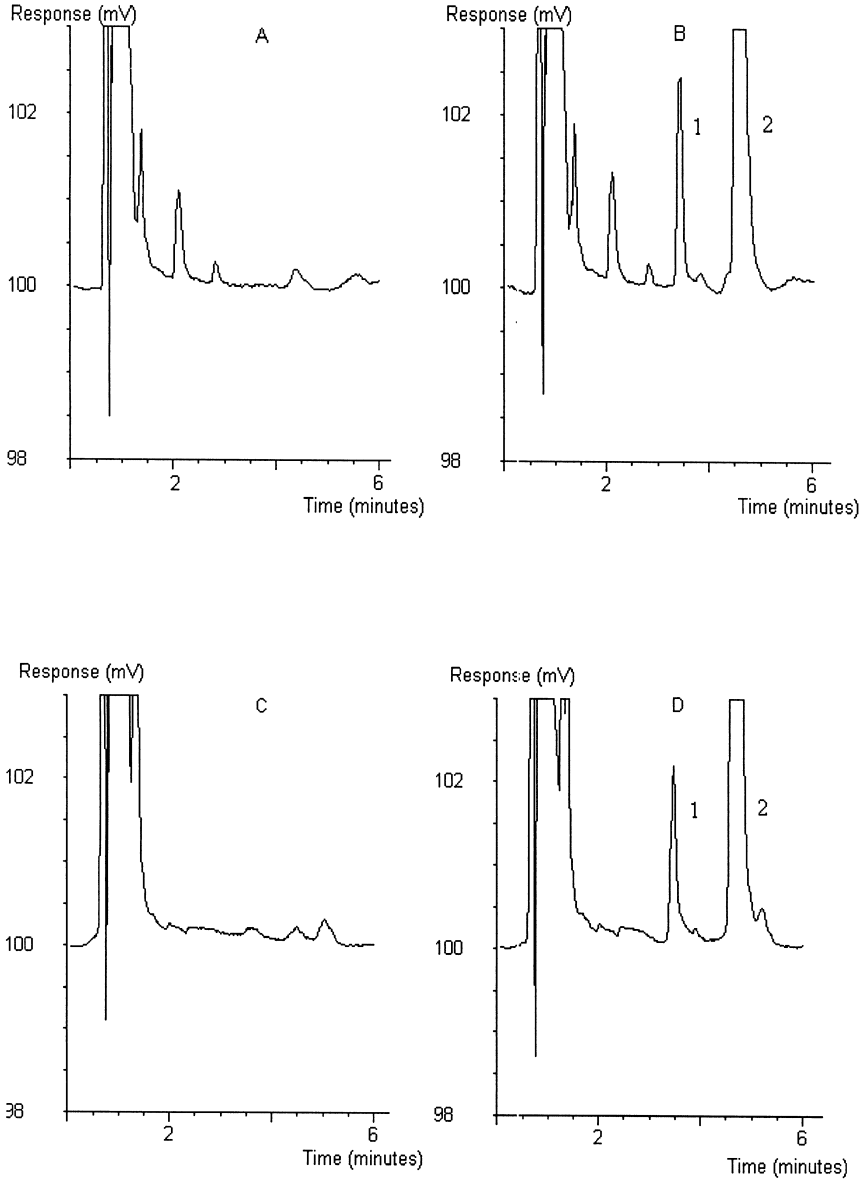


Fig. 4. Representative chromatograms obtained following extraction of AA1 on an Oasis HLB 96-well plate. Peak 1: AA1, peak 2: I.S. (A) Drug-free plasma, (B) plasma spiked with AA1 (500 ng/ml) and I.S., (C) drug-free brain, (D) brain homogenate spiked with AA1 (500 ng/ml).

prepared in drug-free normal human plasma at four different concentrations. The mean accuracy and the precision were satisfactory as shown in Table 2, by far meeting the acceptance criteria: mean accuracy within 85–115% and RSD≤15% (mean accuracy within 80–120% and RSD≤20% at the limit of quantitation). With rufinamide, one calibration sample per column was run at each concentration level. The difference between the two determinations was marginal as depicted by a representative calibration curve in Fig. 6. Over 3 analysis days, the mean slope±SD was 0.711±0.032, the mean intercept and

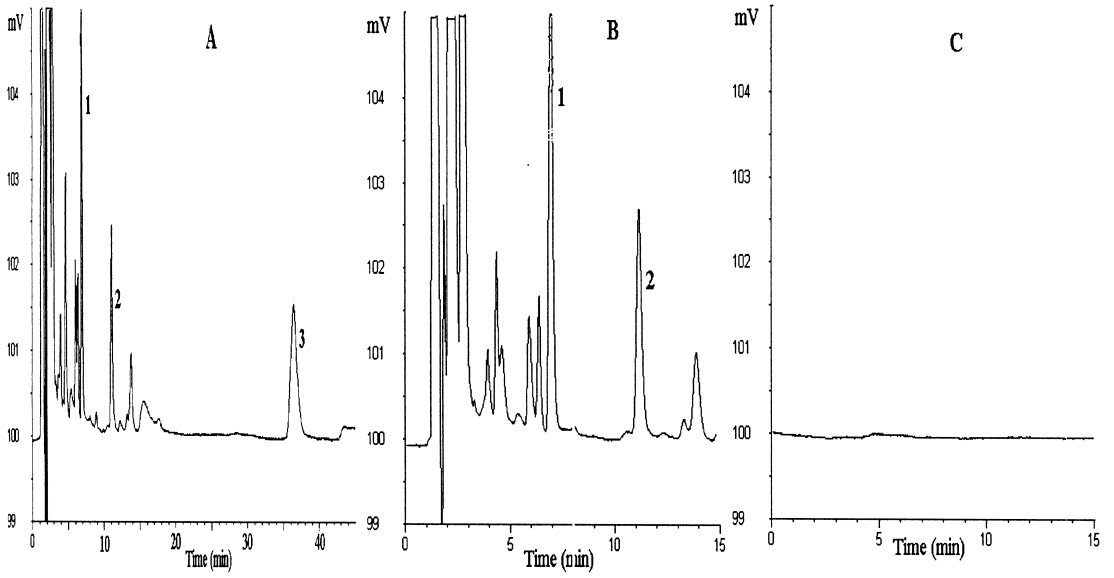


Fig. 5. Representative chromatograms obtained following extraction of rufinamide from a plasma sample taken from a patient given rufinamide and carbamazepine concomitantly (rufinamide concentration determined: 7600 ng/ml). Peak 1: rufinamide, peak 2: I.S., peak 3: carbamazepine. (A) Chromatography using a single column, (B) chromatography obtained on column A using two parallel columns, (C) baseline obtained on column A for a simulated injection, the previous injection on this column corresponding to the extract from a plasma sample taken from a patient given rufinamide and carbamazepine.

coefficient of correlation were 0.009 and 0.995, respectively. In routine analysis, the calibration plasma samples were placed at the beginning of the plate, while quality control samples were placed throughout the plate together with the clinical samples. A good performance across the column was obtained. The accuracy results shown in Table 3 indicate that the accuracy was not column-dependent. The retention times obtained with the two columns were similar and were not modified upon repeated injections.

Because a negative pressure is applied to all wells simultaneously, a few wells may sometimes be eluted less rapidly and less completely than the other wells. This results in a smaller elution volume for these wells. In all cases, the internal standard was found to adequately balance out these differences. As an example, with AA1, for 61 injections of plasma extracts of standards and quality control samples, the mean±SD peak area of the internal standard was 160±14 (range 145–179). One sample resulted in a low area of 90, corresponding nevertheless to a satisfactory accuracy of 108%. This shows that the use of an internal standard is mandatory when 96-

Table 2
Analysis in plasma: intra-day accuracy and precision

Rufinamide			ICL670			AA1		
Nominal concentration (ng/ml)	Accuracy[a] (%)	Precision (RSD, %)	Nominal concentration (ng/ml)	Accuracy[a] (%)	Precision (RSD, %)	Nominal concentration (ng/ml)	Accuracy[a] (%)	Precision (RSD, %)
50	107	9	100	106	6	50	97	11
100	96	10	200	88	4	500	99	3
500	101	6	4000	107	5	1000	94	7
15 000	100	2	40 000	102	3	4000	100	5

[a] Accuracy: (found/nominal concentration)×100 (mean of six replicates).

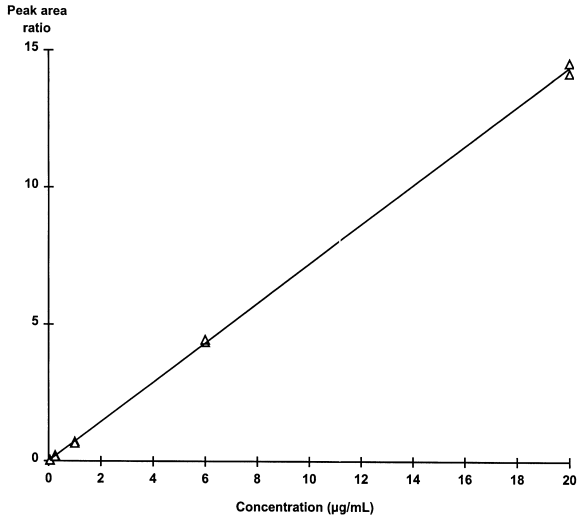


Fig. 6. Representative calibration curve for the determination of rufinamide following SPE extraction. One calibration sample per column was run at each concentration level (correlation coefficient: 0.9998).

well plates are processed with negative pressure, even when using disc plates whose sorbent bed is more uniform than conventional packings.

3.2. Protein precipitation

The potential of protein removal by filtration in the 96-well format was investigated with ICL670, a highly protein-bound (≥99.5%) iron chelator under development. A 3M Empore filter plate PPT containing only the frit used to secure the sorbent bed of a SPE plate was used. Acetonitrile and plasma were sequentially aspirated using an electronic pipette in the dilution mode, separated with an air gap, and subsequently dispensed together into a channel of the filter plate. This permitted a sufficient mixing of acetonitrile and plasma allowing for an adequate effectiveness in precipitating proteins. The ICL670 recovery was calculated from the comparison of the peak areas obtained from spiked plasma samples and spiked blank plasma extracts. The mean recovery was 78%. Linear calibration curves, represented by the plots of the peak area ratio ICL670/I.S. versus ICL670 concentration in the calibration sample, were generated using weighted ($1/x^2$) linear regression (coefficient of correlation >0.99). Five replicate quality control samples were prepared in drug-free normal human plasma at 4 different concentrations.

Table 3
Accuracy of quality control samples in routine analysis for rufinamide

Nominal concentration (ng/ml)	Injection No.	Accuracy[a] (%)		Retention time (min)	
		Column A	Column B	Column A	Column B
100	16		92		7.7
	85	101		7.7	
	86		97		7.8
	87			7.6	
	88	127	105		7.7
500	17	91			7.5
	67	80			7.6
	89	94		7.7	
	90		104		7.7
	91	93		7.6	
15 000	42		90		7.6
	92		100		7.7
	93	101		7.6	
	94		105		7.7
	95	98		7.6	
Mean±SD		98±13	99±6		

[a] Accuracy: (found/nominal concentration)×100.

Table 4
Determination of ICL670 following protein precipitation: intra-day accuracy and precision

Nominal concentration (ng/ml)	Mean accuracy[a] (%)	Precision (RSD, %)
100	87	8
800	102	6
1000	99	3
8000	108	4

[a] Accuracy: (found/nominal concentration)×100. Five replicates were prepared at each concentration.

Table 5
Analysis of AA1 in brain: intra-day accuracy and precision[a]

Nominal concentration (ng/g)	Mean accuracy[b] (%)	Precision (RSD, %)
100	81	16
1000	91	5
2000	107	3
8000	98	2

[a] Calibration standards were prepared in plasma and quality control samples in brain homogenate.
[b] Accuracy: (found/nominal concentration)×100. Six replicates were prepared at each concentration.

The mean accuracy ranged from 87 to 108% with the RSD ranging from 3 to 8% (Table 4). This shows that this simple and fast sample preparation technique can be applied to a highly protein-bound compound with satisfactory reproducibility and may be an alternative to the time consuming manual handling of individual tubes required for centrifugation.

3.3. Extraction from brain

Filtration of the homogenate in the 96-well format using a 3M Empore filter plate was not successful because some wells became blocked. Therefore the proteins were precipitated with NaOH–$ZnSO_4$. The tubes were centrifuged and the supernatant was loaded into the appropriate well of an extraction plate. Some wells became blocked with a 3M Empore C_{18} plate but not with an Oasis HLB plate. With the Oasis plate, the prefilter above the sorbent bed has a pore diameter (20 μm) larger than with the Empore C_{18} plate and consequently must be less prone to clogging. The extraction procedure described for the determination of AA1 in plasma using a C_{18} Empore plate was applied. An Oasis HLB plate containing 10 mg sorbent per well only was selected in order to minimise the volume of eluent. Because rat brain was not available in sufficient amount, calibration standards were prepared in plasma. Quality control samples were prepared in brain homogenate. The chromatograms exhibited in Fig. 4, and the results of accuracy for the brain quality control samples (91–107% and 81% at the limit of quantitation of 100 ng/g) shown in Table 5, indicate that results obtained from spiked plasma or brain were similar. This supports the use of plasma instead of brain to prepare the calibration samples.

3.4. Sample throughput and automation

The time to manually prepare a plate using electronic pipettors was about 1 h for the SPE procedures and 20 min for the protein precipitation procedure. Twelve-channel repeater pipettors were used whenever possible in order to reduce the risk of error and improve productivity. With the rufinamide method, the chromatographic run time was 15 min, thereby allowing the injection of the 96 samples of a plate per day. About 1000 clinical samples were determined using this method, a complete plate being processed on each analysis day. With the ICL670 and AA1 SPE methods, the chromatographic run time was 6–7 min, allowing for the injection of more than 200 samples per day. However, in routine analysis, a plate per day was processed. With the three methods, the pre-columns were exchanged on each analysis day in order to prevent column clogging.

The SPE method for the determination of ICL670 was automated using the Tomtec Quadra 96 system and the Packard Multiprobe II system. The time to prepare a plate with the Tomtec system was only 10–15 min. With the Packard Multiprobe II, it was around 1 h, as when using manual pipettors. A plate is processed unattended with the Packard Multiprobe II system with the exception of the placement of the collection plate, whereas the presence of laboratory personal is necessary with the Tomtec system. Full

automation with a robot such as the Multiprobe II system should be an aid to further increase sample throughput when large series of samples are to be analysed in a short time frame. Method development should be more rapid and more flexible using manual electronic pipettors because the samples corresponding to one or two rows of wells are prepared within 15 min only and the vacuum conditions can be more easily set due to a better visibility of the wells.

4. Conclusion

Empore C_{18} extraction disc plates with low sorbent mass have been successfully employed for the analysis of three drugs with different polarities in plasma in conjunction with HPLC–UV. Ion-pair extraction at a high pH was applied to the most polar compound. The method developed for the assay of this compound in plasma was applied to its determination in brain using an Oasis HLB plate following homogenisation and protein precipitation, thereby saving time for method development. Protein precipitation in the 96-well format was applied to a highly protein-bound iron chelator (>99.5%) with a good recovery. The methods were used routinely, one plate per analysis day being processed, resulting in increase in sample throughput and saving in solvents.

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