ELSEVIER

Journal of Chromatography A, 797 (1998) 251–263

JOURNAL OF CHROMATOGRAPHY A

Direct injection of large volumes of plasma/serum on a new biocompatible extraction column for the determination of atenolol, propranolol and ibuprofen
Mechanisms for the improvement of chromatographic performance

Jörgen Hermansson*, Anders Grahn, Inger Hermansson

ChromTech, Box 6056, 129 06 Hägersten, Sweden

Abstract

Very large volumes of serum/plasma can be directly injected to a new extraction column based on particles with a biocompatible outer surface and C_{18} groups within the pores. The biocompatibility has been obtained by attaching the human plasma protein α_1-acid glycoprotein to the outer surface of the particles. The pores are small enough to exclude the plasma protein molecules. Atenolol and propranolol were extracted on the extraction column as ion-pair with octanesulfonic acid as the counterion. The same counterion was used in the analytical mobile phase. A strong improvement of the recovery can be obtained using octanesulfonic acid as counterion in the extraction mobile phase. The recovery of atenolol increased from about 53.5% to about 93.4% using octanesulfonic acid as counterion. The chromatographic performance was also strongly affected by chromatography of the basic drugs as ion-pair with octanesulfonic acid. The improvement was due to trapping in a smaller section of the extraction column and enrichment of the drug on top of the analytical column. The enrichment was due to the transfer of the analyte to the analytical column in a zone with high concentration of counterion. Furthermore, the sample zone is compressed during the migration on the analytical column. The compression effect was caused by the counterion zone, migrating in front of the sample zone, giving the analyte higher retention on the front side than on the back side of the sample zone. Displacement of protein bound drug (ibuprofen) by addition of octanoic acid, was tested in order to study the influence on the recovery and the effect on the chromatographic performance. The recovery was improved and the chromatographic performance was greatly improved. The improvement obtained on the separation efficiency of ibuprofen was due to enrichment on top of the analytical column and compression during the migration through the analytical column. The enrichment was caused by a reduction of pH in the sample–octanoic acid zone transferred from the extraction column. The octanoic acid zone migrated in front of the sample zone giving a lower pH in front of the ibuprofen zone than behind. Thus, higher retention occurred in front of than behind the sample zone, which gave rise to compression. The methods developed for atenolol, propranolol and ibuprofen could be used for the determination of serum/plasma concentrations after single doses of the drugs with very high accuracy and precision. Linear calibration graphs were obtained and the *r* values were $\geq$0.9999.

Keywords: Ion-pairing reagents; Extraction methods; On-line extraction; Plasma/serum injection; Octanesulfonic acid; Ibuprofen; Atenolol; Propanolol; β-Blockers; Nonsteroidal anti-inflammatory drugs

1. Introduction

Off-line isolation procedures in HPLC bioanalysis

*Corresponding author.

0021-9673/98/$19.00
PII S0021-9673(97)01169-2

is often very time consuming and it also introduces errors in the determination which means that the precision and accuracy can be reduced. This has lead to a great effort among bioanalytical chemists to develop techniques that allow integration of the isolation procedure and the separation step. In principle two different approaches can be used, the first is to use a precolumn coupled to the analytical column via a switching valve and the other possibility is injection of plasma/serum directly on an analytical column, designed for biological samples. Very early, standard columns were tested for direct injection of very small volumes (<5 μl) of plasma/serum [1]. However, standard columns cannot tolerate larger volumes of biological fluids and the chromatographic performance of such columns deteriorates very rapidly. The back pressure also increases after injection of small volumes of biological fluids on such columns. A special type of packing material, the so-called internal surface reversed-phase silica, or mixed-functional silica supports packed in analytical columns demonstrated that it was possible to inject plasma/serum onto the analytical column for direct determination of drugs [2–7]. However, by this technique only smaller amounts of plasma/serum could be injected, which means that low concentration analyses in the low ng/ml range, or even lower, cannot be performed using this approach. The other limitation with this approach is that the concentration of organic modifier that can be used in the mobile phase is limited to 5–10%, depending on the solvent used and the mobile phase pH, otherwise the plasma proteins are precipitated. The other approach, using a precolumn coupled to an analytical column by a switching valve, means that the separation of the analytes from the plasma proteins can be done at conditions that are favourable for the protein molecules. In the analytical step there is no limit to the concentration of organic solvent, nor the pH that can be used on the analytical column. A number of such columns have been described [8–15]. However, the majority of the columns cannot tolerate the injection of large volumes of plasma.

The present paper will discuss the second generation BioTrap column. The column is based on the basic principles developed earlier [15], but the surface chemistry is different. The particles have a biocompatible outer surface and C_{18} groups on the surface within the pores. The biocompatibility has been obtained by attaching the plasma protein α_1-acid glycoprotein (AGP) to the outer surface of the silica particles [15]. The second generation column enables the repetitive injection of very large volumes of plasma, i.e. ~1 ml. However, so far long-term stability studies have been performed by injection of 500-μl serum samples.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile and 2-propanol, of HPLC grade, were obtained from Lab-Scan (Dublin, Ireland). Sodium dihydrogenphosphate (analytical-reagent grade), phosphoric acid (analytical-reagent grade), octanoic acid (>99%) and sodium octylsulfate were obtained from Merck (Darmstadt, Germany). Sodium octanesulfonic acid (98%) was purchased from ICN Biomedicals (OH, USA). Sodium pentanesulfonic acid was obtained from Eastman Kodak (New York, USA). Sodium hydroxide (analytical-reagent grade) was purchased from Eka Nobel (Bohus, Sweden). The drug compounds were obtained from different drug companies.

2.2. Apparatus

The liquid chromatographic system consisted of an LKB 2150 pump (Pharmacia-LKB Biotechnology, Uppsala, Sweden) and an LKB 2248 pump, a Kontron 360 autosampler (Tegimenta, Rotkreuz, Switzerland) equipped with 10-, 200- or 500-μl loops, a C6W six-port switching valve with an electric actuator (Valco, Schenkon, Switzerland), a Shimadzu RF-535 fluorescence detector (Kyoto, Japan), a Spectra 100 variable-wavelength UV detector (Spectra-Physics, San Jose, CA, USA) and a Perkin-Elmer Series 200 refractive index detector (San Jose, CA, USA). All experimental data were collected and analysed on a Kontron 450 MT2 data system (Eching/Munich, Germany), which also controlled the autosampler and the switching valve.

The following columns were used in the study: CT-Sil C_{18}, 100×4.6 mm I.D., 5 μm (ChromTech, Hägersten, Sweden); Hypersil Elite C_{18}, 150×4.6

mm I.D., 5 μm and Hypersil Elite C_{18} guard columns (10×4.0 mm I.D., 5 μm), (Hypersil, UK); Zorbax SB-CN, 150×4.6 mm I.D., 5 μm and Zorbax SB-CN guard columns (12.5×4.6 mm I.D., 5 μm); (Rockland Technologies, USA).

The extraction column was BioTrap 500 C_{18}, 20×4.0 mm I.D. (ChromTech). The filter holder in polyether ether ketone (PEEK) and the 2-μm filter, used in front of the extraction column, were obtained from ChromTech.

2.3. Chromatographic system

Fig. 1 shows schematic diagrams of the chromatographic system used. The extraction column is the second generation BioTrap 500 C_{18} (20×4.0 mm). The column is coupled to the analytical column through a six-port switching valve as shown in Fig. 1. In the extraction position the samples are injected by the autosampler. The samples are pumped onto the extraction column by the extraction mobile phase (pump A). The plasma proteins are eluted to waste whereas the drug compounds (and other low-molecular-mass compounds) are enriched within the pores of the particles. When the valve is switched to the elution position the drug compounds are eluted (backflushing of the extraction column) onto the analytical column by the analytical mobile phase (pump B). The valve is again switched to the extraction position in order to reequilibrate the extraction column with the extraction mobile phase, prior to the next injection. Simultaneously, the final separation on the analytical column is completed.

A 2-μm on-line filter is inserted in the chromatographic system between the autosampler and the six-port valve (Fig. 1), in order to remove particles from the biological samples. A guard column is used to protect the analytical column from particles and endogenous compounds which are bound to the analytical column with high affinity.

2.4. Determination of atenolol, propranolol and ibuprofen

Detailed descriptions of the extraction and the analytical mobile phases, columns and switching times of the six-port valve, flow-rates etc. are given in the legends to the chromatograms for the three compounds.

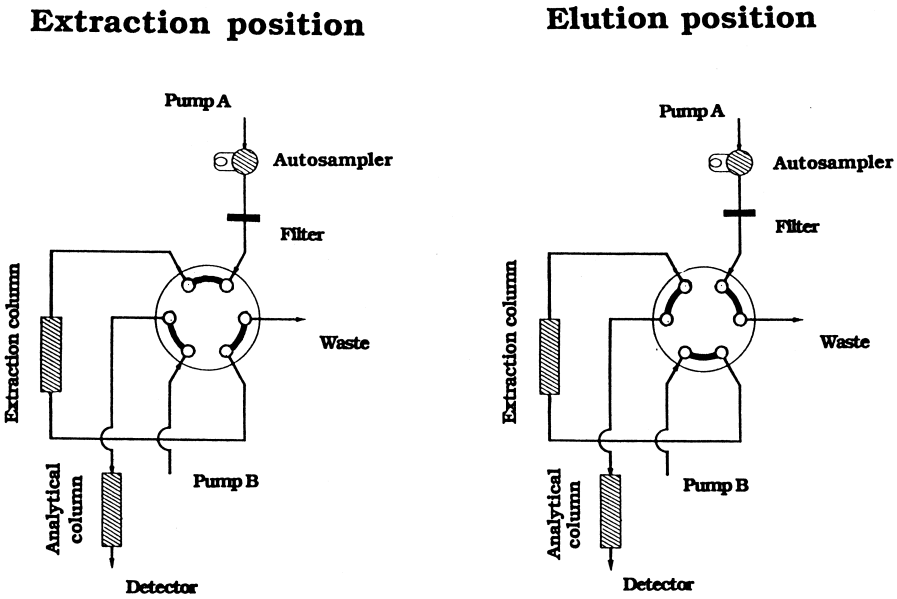

Fig. 1. Schematic illustration of the chromatographic system.

2.5. Preparation of serum samples and validation procedures

The serum samples were stored at −20°C. Standard samples were prepared by adding known amounts of the drug compounds to an exact volume of serum. Prior to injection, the samples were centrifuged for 7 min (Hettich Mikroliter centrifuge, Tuttlingen, Germany) at 15 000 rpm (11|300 *g*), in order to remove cryoproteins and other particulate impurities. The absolute recoveries were determined by addition of known amounts of the different drug compounds to blank serum and to the analytical mobile phase. The serum samples were injected onto the extraction column and the samples dissolved in the mobile phase were injected directly onto the analytical column. The absolute recovery was calculated by dividing the peak area obtained for the serum samples with that obtained for the samples dissolved in the mobile phase and injected directly onto the analytical column.

3. Results and discussion

The extraction column used in this study is the second generation BioTrap column. The column makes possible the direct and repetitive injection of very large volumes of serum/plasma as will be demonstrated below. The biocompatibility of the extraction column has been achieved by covering the outer surface of the particles with the extremely stable plasma protein, AGP [16]. The inner surface of the particles contains C_{18} groups. The pores of the particles are small enough to exclude plasma proteins and other macromolecules, whereas low-molecular-mass compounds are able to penetrate the pores and be adsorbed onto the hydrophobic inner surface of the particles [14]. A schematic drawing of a particle can be seen in Fig. 2.

3.1. Extraction step

The extraction column has been used in the chromatographic system, as described in Section 2, for the determination of drugs in serum samples. In this study, three drugs of different character have been used as model compounds; the β-blockers

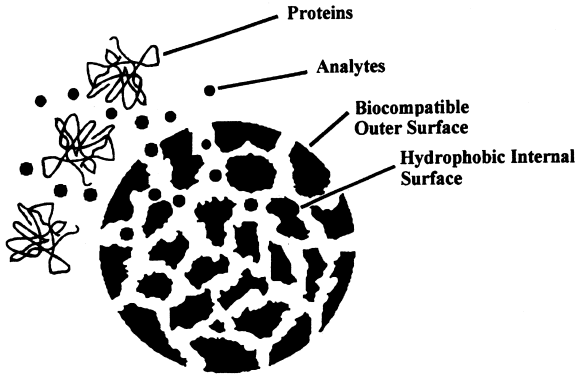

Fig. 2. Schematic drawing of a particle.

propranolol (amine, pK_a~9.5) and atenolol (amine, pK_a~9.6) and the nonsteroidal anti-inflammatory drug ibuprofen (carboxylic acid, pK_a~4.5).

In order to create enough selectivity in the system and to get high recoveries of the drugs in the extraction step, it is necessary to optimize the extraction and the analytical mobile phases as well as the switching times of the valve.

The on-line extraction step involves the separation of proteins and low-molecular-mass compounds and enrichment of the drug compound(s) on the extraction column. The extraction mobile phase must therefore have a composition which will give the drug compounds high affinity to the internal hydrophobic phase and is compatible with the plasma proteins. Consequently, the choice of buffer salts, pH, and type and concentration of organic modifier are of crucial importance. It has been found that it is favourable to remove serum proteins using a pH which is close to the physiological pH, i.e. around neutral and also limit the concentration of organic solvent in the extraction mobile phase. 2-Propanol is normally used at a concentration of ≤5% and the acetonitrile and the methanol concentrations are limited to ≤10%. It has also been found that it is preferable to use phosphate buffers in the extraction mobile phase, due to the compatibility with the serum proteins. Fig. 3 demonstrates the elution profile of a 500-μl blank serum sample from the extraction column, detected at 280 nm, with a mobile phase of 4% 2-propanol in 20 m*M* sodium phosphate buffer (pH 7.0). After ~4 min, the serum proteins

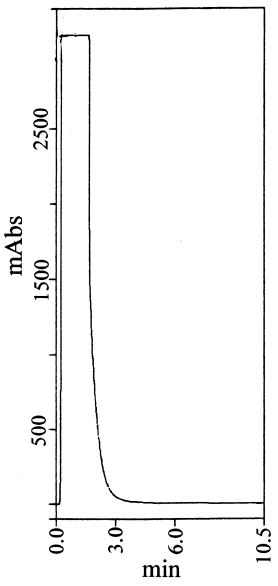

Fig. 3. Elution profile of a 500-μl blank serum sample from the extraction column. Chromatographic conditions: extraction column, BioTrap 500 C_{18} (20×4.0 mm I.D.); mobile phase, 4% 2-propanol in 20 m*M* sodium phosphate buffer (pH 7.0); flow-rate, 0.80 ml/min; UV detection at 280 nm.

and hydrophilic low-molecular-mass endogenous compounds are eluted from the extraction column using a flow-rate of 0.8 ml/min. The extraction can be performed at higher flow-rate in order to shorten the extraction time.

Two of the compounds used in the study are protein bound to a high degree. Propranolol is protein bound to ~95% and ibuprofen to ~99% [17]. The degree of protein binding affects the chromatographic properties of the solutes on the extraction column, when serum samples are injected, as is demonstrated below.

The retention of a solute, s, on a reversed-phase column can be expressed by

$$k'_s = qD_s \quad (1)$$

where k'_s is the retention factor of the solute s, q is the phase ratio on the column and D_s is the distribution ratio of the solute. If it is assumed that the solute s is preferentially bound to one binding site on one major binding protein P, and the protein and the drug–protein complex, PS, is excluded from the pores and the binding of the protein to the external surface of the particles can be neglected, the distribution ratio of s is given by

$$D_s = [SA_s]/[S] + [PS] \quad (2)$$

where [S] and [PS] are the concentrations of the free drug and the drug–protein complex, respectively. $[SA_s]$ is the concentration of the drug bound to the hydrophobic binding site, A_s, in the pores of the particles. By combining Eqs. (1) and (2), an expression for the retention of a solute in a sample containing proteins can be derived

$$k'_{sp} = k'_s/(1 + [PS]/[S]) \quad (3)$$

where [PS]/[S] is the drug–protein binding ratio and k'_{sp} and k'_s are the retention of the solute with or without protein in the sample zone.

If the drug has a high drug–protein-binding ratio, ([PS]/[S]), and/or low affinity to the hydrophobic inner surface, i.e. a low k'_s ratio, the solute must migrate a long distance on the extraction column in order to be extracted quantitatively. With a short extraction column the recovery will be too low. The recovery can be improved by two different approaches, reduction of the drug–protein binding ratio ([PS]/[S]) and increasing the affinity of the drug to the internal surface of the particles (higher k'_s ratio). The drug–protein binding ratio can be reduced by:

1. Addition of a displacer that will compete with and displace the drug from the plasma proteins (organic solvent, compound with a structure similar to the analyte)
2. Changing the protein conformation in a way that the affinity of the drug is reduced (changing the pH)
3. Dilution of the sample; the effect obtained by dilution is dependent on the total concentration of both the protein and the drug, as well as of the binding affinity of the drug.

The affinity of the drug to the internal surface can be affected by the surface chemistry and by adjusting the extraction mobile phase in a way that give higher k'_s ratio. The k'_s ratio can be affected by:

1. Addition of an ion-pair agent to the extraction mobile phase
2. Adjusting the pH
3. Adjusting the organic modifier concentration.

In the present paper we have studied both the reduction of the drug– protein binding ratio by utilizing the displacement approach and increasing the affinity of the drug to the internal surface of the particles, the k'_s ratio, by utilizing ion-pair distribution.

3.2. Extraction by ion-pair distribution

Atenolol is protein bound to a low degree compared to propranolol. It has a much more hydrophilic character than propranolol, giving a relatively low k'_s ratio, which is the major reason for the low recovery obtained for atenolol using an extraction mobile phase of 2% 2-propanol in 30 m*M* sodium phosphate buffer (pH 7.0). However, by adding 5 m*M* of the ion-pairing agent sodium octylsulfate to the extraction mobile phase, the recovery of atenolol was improved to 93.4% ($n=3$, R.S.D.=0.6%) by injection of 200 μl serum. The recovery of atenolol without the ion-pairing agent was 53.5% ($n=3$, R.S.D.=2.1%). It is interesting to note that this change of the extraction mobile phase induces such a strong improvement of the recovery despite the relatively large injection volume. This is most likely due to the low degree of protein binding.

Propranolol is a β-blocker which is much more hydrophobic than atenolol and it has a high degree of protein binding ~95% [17]. Using an extraction mobile phase of 4% 2-propanol in 20 m*M* sodium phosphate buffer (pH 7.0) gave a recovery of 93.6% ($n=3$, R.S.D.=1.2%). In an attempt to improve the recovery 5 m*M* sodium octanesulfonic acid was added to the extraction mobile phase. However, the recovery was only slightly improved by addition of the alkylsulfonic acid. This is probably because of the high degree of protein binding of propranolol compared to atenolol and the fact that as much as 500 μl serum was injected.

3.3. Effect of displacement on the recovery

Ibuprofen is a carboxylic acid and therefore it seems reasonable to perform the extraction of ibuprofen at the lowest pH that the column can withstand. However, by direct injection of plasma it is favourable to remove the plasma proteins at conditions that are close to the physiological conditions, i.e, a pH close to 7.4. Despite that fact and for comparison, the recovery of ibuprofen was studied using a mobile phase of 2% 2-propanol in 30 m*M* sodium phosphate buffer (pH 3.0). This pH reduces the charge of the analyte and thereby increases the affinity of ibuprofen to the hydrophobic inner surface of the extraction column. Owing to the relatively high concentrations of ibuprofen in serum and the use of a sensitive and selective fluorimetric detection method, it was possible to inject only 10 μl serum for determination of concentrations obtained after administration of a single dose of ibuprofen. A recovery of 91.0% was obtained by this method.

Ibuprofen and other nonsteroidal anti-inflammatory drugs (NSAIDs) are strongly bound to human serum albumin (HSA). Ibuprofen is protein bound to 99% at physiological conditions. Different types of fatty acids can affect the binding properties of NSAIDs to HSA [18]. Therefore, the influence of a displacer, competing with ibuprofen for the protein binding, was studied by adding 5 or 10 m*M* octanoic acid to a mobile phase of 2% 2-propanol in 30 m*M* sodium phosphate buffer (pH 7.0). The results are summarized in Table 1. The highest recovery (91.0%) was obtained using an extraction mobile phase with a pH of 3.0. As expected, the recovery of ibuprofen decreased when increasing the extraction pH up to 7.0, due to the increase of the degree of charge of the analyte. It is reasonable to assume that ibuprofen is retained as the sodium ion-pair at this pH. A recovery of 77.7% was obtained at pH 7.0. By adding 5 m*M* octanoic acid to the mobile phase the recovery was improved to 88.6%, indicating that octanoic acid has displaced ibuprofen from the plasma proteins, giving a larger free fraction which can be adsorbed onto the hydrophobic internal surface. In order to elucidate to what degree the higher sodium ion concentration (in the extraction mobile phase containing octanoic acid) affected the recovery, due to ion-pair distribution, a mobile phase of 2% 2-propanol in 90 m*M* sodium phosphate buffer (pH 7.0) was used. Compared to the mobile phase containing 30 m*M* buffer, only a slight improvement of the recovery was observed, from 77.7% to 80.5% despite the fact that the sodium concentration was three times higher than in the system with octanoic acid. Therefore the displace-

Table 1
Performance of the chromatographic system with different composition of the extraction mobile phase

Extraction mobile phase	t_R (min)	$W^a_{1/2}$ (min)	$N^b_{apparent}$	Recovery[d] (%)
[c]	1.62	0.092	–	–
2% 2-propanol in 30 m*M* sodium phosphate (pH 3.0)	7.05	0.111	22 190	91.0
2% 2-propanol in 30 m*M* sodium phosphate (pH 7.0)	7.01	0.117	19 830	77.7
2% 2-propanol in 30 m*M* sodium phosphate with 5 m*M* octanoic acid (pH 7.0)	7.62	0.085	44 230	88.6
2% 2-propanol in 30 m*M* sodium phosphate with 10 m*M* octanoic acid (pH 7.0)	8.06	0.077	60 660	87.7

Sample: 10 μl serum containing 19.5 μg/ml of ibuprofen.
Other conditions: see the legend to Fig. 7.
[a] The peak width measured at half height.
[b] Calculated according to: $N_{apparent} = 5.54\ (t_R \text{ (coupled column system)}/W_{1/2})^2$.
[c] Direct injection onto the analytical column. Ibuprofen (19.5 μg/ml, 10 μl inj. vol.) dissolved in the analytical mobile phase.
[d] $n = 3$, R.S.D. < 1.2% in all cases.

ment of ibuprofen by octanoic acid is the chief reason for the increased recovery.

3.4. Chromatographic performance and enrichment on the analytical column

In the analytical step the adsorbed solute and endogenous compounds are transferred from the extraction column to the analytical column by the analytical mobile phase. The composition of the analytical mobile phase is chosen in a way that enough selectivity can be created in the final separation of the low-molecular-mass compounds on the analytical column. It helps if the analytical mobile phase has the ability to rinse the extraction column of endogenous compounds with very high affinity to the hydrophobic inner surface of the extraction column, prior to the next injection. To obtain sharp peaks, it is advantageous if the combination of the extraction and the analytical mobile phases produce the prerequisites for obtaining enrichment of the solute on the analytical column. A rough estimate of the enrichment effect in a column switching system is given by the following equation [19]:

$$V_e = V_i(1 + k')^{-1} \quad (4)$$

where V_e is the effective injected volume on the analytical column, V_i is the volume of sample transferred from the extraction to the analytical column and k' is the capacity ratio of the solute on the analytical column using the first mobile phase. However, if the analyte is extracted as ion-pair with a hydrophobic counterion like octanesulfonic acid, the V_e value that is calculated according to Eq. (4) is too high. The reason for the high V_e value is that a large amount of the hydrophobic counterion is adsorbed onto the extraction column during equilibration. When the valve is switched and the analytical mobile phase (containing a high concentration of organic solvent) starts to be pumped through the extraction column (backflushing), the analytical mobile phase elutes the hydrophobic counterion from the extraction column in a zone together with the analyte and the extraction mobile phase components. The high concentration of counterion gives a much higher capacity ratio and thereby a smaller V_e value than calculated according to Eq. (4).

3.5. Atenolol

Table 2 shows a comparison between the efficiency (expressed as peak width) obtained by direct injection of a 10-μl sample of atenolol (dissolved in the analytical mobile phase) onto a cyanopropyl column, and the efficiency obtained by using the coupled column system and injecting a 200-μl serum sample. As demonstrated in Table 2, the peak width ($W_{1/2}$) generated in the coupled column system, is nearly the same as by direct injection of only 10 μl onto the analytical column. One of the reasons for obtaining these sharp peaks in the coupled column

Table 2
Comparison of column efficiency, with and without extraction column

	$W^{a}_{1/2}$ (min)
Coupled column system[b]	0.094
Analytical column[c]	0.091

Sample: atenolol; other conditions: see the legend to Fig. 4.
[a] The peak width measured at half height.
[b] A 200-μl volume of serum injected (126 ng/ml).
[c] Direct injection of a 10-μl sample (1.16 μg/ml), dissolved in the analytical mobile phase, onto the analytical column.

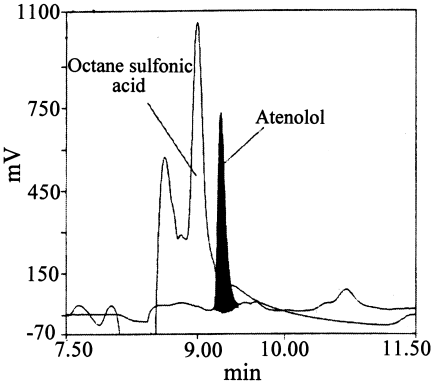

Fig. 4. Overlay plot obtained from injection of a serum sample containing 125 ng/ml of atenolol (fluorescence detection) and from injection of the extraction mobile phase (RI-detection). Chromatographic conditions: injection volume, 200 μl; extraction column, BioTrap 500 C_{18} (20×4.0 mm I.D.); extraction mobile phase, 2% 2-propanol in 30 m*M* sodium phosphate buffer with 5 m*M* sodium octanesulfonic acid (pH 7.0); flow-rate, 0.80 ml/min; analytical column, Zorbax SB-CN (150×4.6 mm I.D., 5 μm) and a Zorbax SB-CN guard column (12.5×4.6 mm I.D., 5 μm); mobile phase, 25% acetonitrile in 30 m*M* sodium phosphate buffer with 2 m*M* sodium octanesulfonic acid (pH 3.0); flow-rate, 1.0 ml/min; fluorimetric detection (excitation wavelength 230 nm, emission wavelength 300 nm) and refractive index detection; switching times of the six-port valve, extraction position 6 min (sample extraction), elution position 6 min (transfer of analyte), extraction position 4 min (reequilibration of the extraction column).

system, is that atenolol is trapped effectively in the extraction column due to the ion-pair extraction, giving a smaller starting zone when the analyte is eluted onto the analytical column. Furthermore, the combination of the extraction and the analytical mobile phases give the prerequisites for obtaining an enrichment effect on the analytical column. The extraction mobile phase consisted of 2% 2-propanol in 30 m*M* sodium phosphate buffer with 5 m*M* sodium octylsulfate (pH 7.0), whereas the analytical mobile phase consisted of 25% acetonitrile in 30 m*M* sodium phosphate buffer with 2 m*M* sodium octylsulfate (pH 3.0). The higher concentration of the ion-pairing agent in the extraction mobile phase, together with the ion-pairing agent stripped off from the hydrophobic inner surface of the extraction column by the analytical mobile phase, means that atenolol is eluted from the extraction column in a zone with a high concentration of counterion, which gives rise to enrichment on top of the analytical column.

The system was also tested with some other counterions in order to study if the enrichment effect still remained. By exchanging sodium octylsulfate in both the extraction and the analytical mobile phases with the same concentrations of sodium octanesulfonic acid, the chromatograms were almost identical.

Another factor which might influence the separation efficiency of the atenolol peak is that atenolol is eluted through the analytical column in a zone of decreasing counterion concentration, which makes the tail of the atenolol zone migrate faster than the front, compressing the peak. This is demonstrated by the chromatograms in Fig. 4. One chromatogram demonstrates injection of 200 μl serum containing atenolol using fluorescence detection. The other demonstrates the injection of the extraction mobile phase using RI detection, which means that octanesulfonic acid could be detected. The chromatograms show that there is an overlap of the atenolol and the octanesulfonic acid peaks. The overlap was even larger when they were transferred from the extraction column onto the top of the analytical column. The above facts support the assumption that compression contributes to the high separation efficiency obtained for atenolol. Peak compression effects caused by system peaks on analytical columns have been reported [20].

If sodium octanesulfonic acid was replaced with the more hydrophilic counterion sodium pentanesulfonic acid in the extraction mobile phase, the atenolol peak became much broader. There might be more than one reason behind this finding. However, with this more hydrophilic counterion, it is very

likely that atenolol will be spread over a larger section of the extraction column, which is supported by the lower recovery obtained with sodium pentanesulfonic acid. Furthermore, as pentanesulfonic acid is more hydrophilic than the octane analog, it is less retained. This means that the pentanesulfonic acid zone could be separated from the atenolol zone and thereby no compression should be obtained on the analytical column. The final chromatographic method used for the determination of atenolol in serum is demonstrated in Fig. 5.

3.6. Propranolol

The influence of the extraction and the analytical mobile phase compositions on the chromatographic performance of propranolol have been studied using two different extraction mobile phases, 4% 2-propanol in a 20 m*M* phosphate buffer (pH 7.0) with or without 5 m*M* sodium octane sulfonic acid. Two different analytical mobile phases were also used, 28% acetonitrile in a 116 m*M* phosphate buffer (pH 2.8) and 33% acetonitrile in 116 m*M* phosphate buffer (pH 2.8) and 2 m*M* sodium octane sulfonic acid. A 500-μl volume of serum containing 12.2 ng/ml was injected. The studies were performed in order to elucidate the prerequisites for obtaining enrichment and compression of the solutes in line with the findings for atenolol.

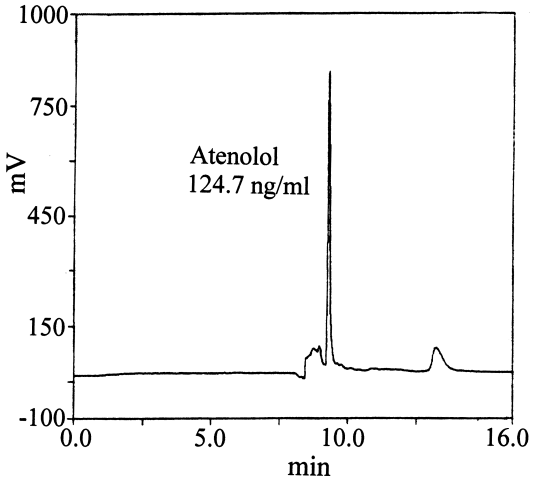

Fig. 5. Isolation of atenolol from spiked human serum. Chromatographic conditions: fluorimetric detection (excitation wavelength 230 nm, emission wavelength 300 nm), other conditions, see Fig. 4.

3.6.1. Ion-pair agent in the extraction mobile phase

The effect on the chromatographic performance of sodium octanesulfonic acid in the extraction mobile phase was studied. No sodium octanesulfonic acid was used in the analytical mobile phase. The peak width at the half height of the peak, ($W_{1/2}$), was reduced from 0.280 to 0.219 min by addition of 5 m*M* sodium octanesulfonic acid to the extraction mobile phase. The peak width obtained by direct injection of only 10 μl propranolol sample (dissolved in the analytical mobile phase) onto the analytical column, was 0.196 min. This is only slightly better than that obtained by injection of 500 μl serum, with sodium octanesulfonic acid in the extraction mobile phase, in the coupled column system. A slight increase of the retention time of propranolol was also observed, which is most likely due to the transfer of octanesulfonic acid from the extraction to the analytical column, which means that ion-pair distribution of propranolol affects the retention. The analytical mobile phase can effectively wash out the octanesulfonic acid from the extraction column, due to the high concentration of acetonitrile (28%). The improvement of the efficiency is most likely due to enrichment on top of the analytical column, due to the high concentration of counterion in the zone transferred from the extraction column. It is not likely that the improvement of the separation efficiency is caused by trapping of propranolol more effectively in the extraction column when using octanesulfonic acid as counterion. This is supported by the fact that the recovery of propranolol was not increased with sodium octanesulfonic acid in the extraction mobile phase.

Addition of sodium octanesulfonic acid to the extraction mobile phase increased the recovery of endogenous compounds, although the recovery of propranolol was unaffected. However, despite the higher recovery of endogenous compounds, the system with sodium octanesulfonic acid in the extraction mobile phase generated very good selectivity for propranolol with no interfering peaks.

3.6.2. No ion-pair agent in the extraction mobile phase

Chromatography of propranolol with sodium octanesulfonic acid in the analytical mobile phase but not in the extraction mobile phase gives no enrichment effect. Obviously the retention of the solute is not high enough on the analytical column, when it is transferred in a zone of the extraction mobile phase.

3.6.3. Ion-pair agent in both the extraction and the analytical mobile phase

The best chromatographic performance was obtained with 5 m*M* sodium octanesulfonic acid in the extraction mobile phase and 2 m*M* in the analytical mobile phase. This combination of mobile phases gives enrichment of propranolol on the analytical column according to the principles discussed above and also a constant retention. Therefore these mobile phases were used in the final method for propranolol (see Fig. 6).

3.6.4. Compression of the sample zone

The separation efficiency of atenolol was affected in three different ways by adding octanesulfonic acid to the extraction mobile phase; more effective trapping in the extraction column, enrichment on top of the analytical column and compression of the sample zone during the migration on the analytical column. In order to study whether propranolol was also compressed by the counterion zone, the extraction mobile phase was injected and RI detection was used. The overlay chromatograms demonstrated that the octanesulfonic acid peak is well resolved from the propranolol peak. It can therefore be concluded that the propranolol zone and the counterion zone enter the column together. However, the bands separate after a short migration and compression does not influence the width of the zone to any significant extent. This means that the improved separation efficiency of propranolol is caused by more effective enrichment on top of the analytical column.

3.7. Ibuprofen

The most interesting effects on the chromatographic properties of ibuprofen were found on adding octanoic acid to the extraction mobile phase,

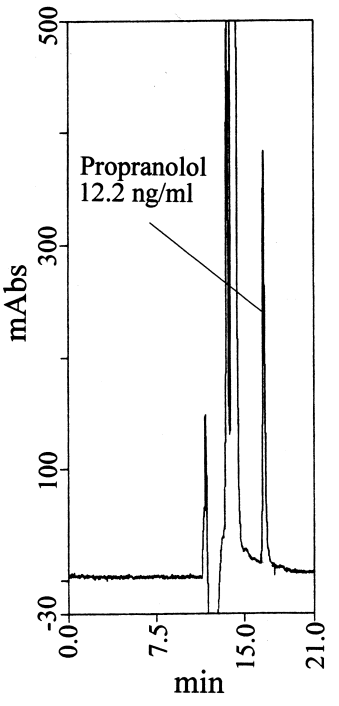

Fig. 6. Isolation of propranolol from spiked human serum. Chromatographic conditions: injection volume, 500 μl; extraction column, BioTrap 500 C_{18} (20×4.0 mm I.D.); extraction mobile phase, 4% 2-propanol in 20 m*M* sodium phosphate buffer with 5 m*M* sodium octanesulfonic acid (pH 7.0); flow-rate, 0.80 ml/min; analytical column, Hypersil Elite C_{18} (150×4.6 mm I.D., 5 μm) and a Hypersil Elite C_{18} guard column (10×4.0 mm I.D., 5 μm); mobile phase, 33% acetonitrile in 116 m*M* sodium phosphate buffer with 2 m*M* sodium octanesulfonic acid (pH 2.8); flow-rate, 1.0 ml/min; fluorimetric detection (excitation wavelength 220 nm, emission wavelength 340 nm); switching times of the six-port valve, extraction position 10 min (sample extraction), elution position 6 min (transfer of analyte), extraction position 5 min (reequilibration of the extraction column).

see Table 1. Addition of 5 m*M* octanoic acid improved the recovery from 77.7% to 88.6%. Furthermore, a great improvement of the efficiency was also obtained, as well as an increase in the retention of ibuprofen. As shown in Table 1, the peak width ($W_{1/2}$) decreased from 0.12 to 0.085 min, whereas the retention increased from 7.01 to 7.62 min with 5 m*M* octanoic acid in the extraction mobile phase. Decrease in the retention of ibuprofen, due to competition between octanoic acid and ibuprofen for binding to the stationary phase of the analytical column would be expected. When the concentration

of octanoic acid was increased to 10 m*M* in the extraction mobile phase, a further increase in efficiency was seen and the retention time increased to 8.06 min, whereas the recovery was only affected to a limited extent. The separation efficiency obtained by direct injection of a 10-μl sample of ibuprofen (dissolved in the analytical mobile phase) onto the analytical column is lower than the efficiency generated in the coupled column system, with octanoic acid in the extraction mobile phase, see Table 1. Fig. 7 shows a chromatogram of a serum sample containing ibuprofen, obtained in the column switching system with 10 m*M* octanoic acid in the extraction mobile phase.

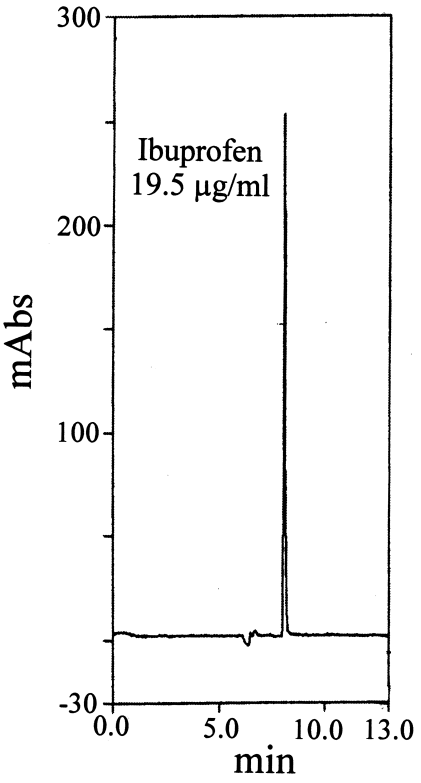

Fig. 7. Isolation of ibuprofen from spiked human serum. Chromatographic conditions: injection volume, 10 μl; extraction column, BioTrap 500 C_{18} (20×4.0 mm I.D.); extraction mobile phase, 2% 2-propanol in 30 m*M* sodium phosphate buffer with 10 m*M* octanoic acid (pH 7.0); flow-rate, 0.80 ml/min; analytical column, CT-Sil C_{18} (100×4.6 mm I.D., 5 μm); mobile phase, 35% acetonitrile in 30 m*M* sodium phosphate buffer (pH 7.0); flow-rate, 1.0 ml/min; fluorimetric detection (excitation wavelength 225 nm, emission wavelength 535 nm); switching times of the six-port valve, extraction position 5 min (sample extraction), elution position 4 min (transfer of analyte), extraction position 4 min (reequilibration of the extraction column).

To elucidate the background for the above described findings a UV detector operating at 200 nm was coupled to the analytical column. A very large octanoic acid peak appeared in the chromatogram on injection of the extraction mobile phase containing octanoic acid, whereas almost no peaks could be detected without octanoic acid. Both with 5 m*M* and 10 m*M* octanoic acid it was found that the ibuprofen peak eluted at the end of the octanoic acid peak, most likely in a gradient of decreasing concentration of octanoic acid. This is illustrated by the overlay plots shown in Fig. 8, where the chromatograms of the octanoic acid zone, detected at UV 200 nm, are compared with the ibuprofen peak detected by fluorimetric detection. A reasonable explanation to the increased retention of ibuprofen using an extraction mobile phase containing octanoic acid, is that the buffer capacity of the analytical mobile phase is not high enough to keep the pH at 7.0. A decreasing pH in the octanoic acid zone will cause an increase of the retention. By collecting fractions eluting from the analytical column, it was found that the pH of the mobile phase was lowered in the zone of octanoic acid. A likely explanation of the sharp

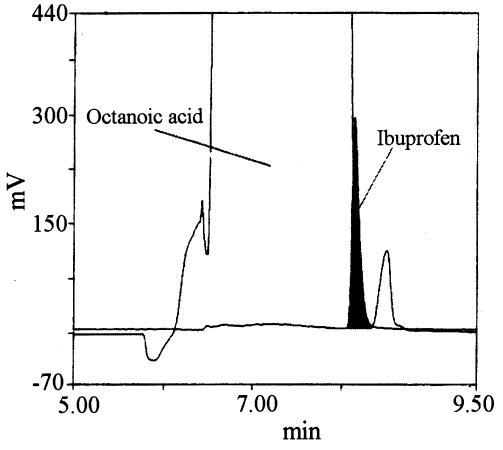

Fig. 8. Overlay plot obtained from injection of a serum sample containing 19.5 μg/ml of ibuprofen (fluorescence detection) and from injection of the extraction mobile phase (UV detection). Chromatographic conditions: fluorimetric detection (excitation wavelength 225 nm, emission wavelength 535 nm) and UV detection (200 nm); other conditions, see Fig. 7.

peaks obtained in the column switching system, with octanoic acid in the extraction mobile phase, can also be derived from the results above. When ibuprofen enters the analytical column in a zone with high concentration of octanoic acid, and thereby in a zone with a lower pH than the mobile phase, an enrichment effect can be obtained, due to decreased charge of ibuprofen in the zone. Furthermore, as shown in Fig. 8, ibuprofen is eluted from the analytical column in a zone of decreasing concentration of octanoic acid and thereby in a zone with a gradient of increasing pH. With a higher pH at the tail of the peak, there are prerequisites for compression of the sample zone, as the tail migrates faster than the front of the peak. Due to the higher efficiency obtained in the system with octanoic acid in an extraction mobile phase of pH 7.0, this approach seems to be favourable compared to the method where a pH of 3.0 was used.

3.8. Stability of the extraction column

The stability of the system was studied using the atenolol as well as the propranolol method. Using the propranolol method serum samples (500 μl) containing about 10 ng/ml were injected. The system demonstrated very good performance throughout the study. Two hundred samples (500 μl) were injected, corresponding to 100 ml of serum. The guard column in front of the analytical column was exchanged after injection of about 60–65 serum samples corresponding to injection of 30 to 32.5 ml serum. A slight increase of the pressure was observed over the guard column when it was exchanged. The efficiency was also slightly affected. However, replacing the analytical guard column totally restored the system. The recovery and the retention were constant and no increase in pressure was observed over the extraction column during the study. The same extraction column was used throughout the study.

The stability of the atenolol method was studied by injection of 108 200-μl serum samples. The system worked excellently throughout the study. The recovery, the retention time as well as the pressure over the extraction column were constant. A new guard column was inserted after 68 serum samples due to a slight increase of the pressure over the guard. However, the chromatographic performance was not affected by the guard column at that time and the pressure over the analytical column was unaffected.

3.9. Validation of the methods

Fully automated methods for quantitative determination of atenolol, propranolol and ibuprofen are described in the present paper and they have demonstrated excellent properties. The standard curves are linear in the concentration range found in serum after administration of a single dose or during therapy. The concentration ranges studied for propranolol, atenolol and ibuprofen were, 1–25 ng/ml, 5–200 ng/ml and 0.5–40 μg/ml, respectively. Linear regression equations of the calibration graphs for propranolol, atenolol and ibuprofen are given below:

$$Y_{\text{propranolol}} = -1.444 + 8.631x$$

$$Y_{\text{atenolol}} = -0.0761 + 0.6450x$$

$$Y_{\text{ibuprofen}} = 0.0672 + 1.444x$$

The r values are in all cases better than 0.9999. No internal standard is needed with this kind of method.

The intra- and inter-day precisions were also determined. The intra-day precisions for propranolol (12.2 ng/ml, $n=6$), atenolol (126 ng/ml, $n=6$) and ibuprofen (1.98, 19.9 μg/ml, $n=6$) were, 0.61, 0.47, 0.87 and 0.26%, respectively. The inter-day precisions for propranolol (12.2 ng/ml, $n=6$), atenolol (126 ng/ml, $n=6$) and ibuprofen (17.9 μg/ml, $n=7$) were, 1.27, 1.52 and 0.68%, respectively. The precision is very high, probably because this kind of method has no off-line manipulations with the serum samples. The precision is determined by the integration process and the precision of the autosampler.

As has been reported in Section 3.1, the recovery of atenolol, propranolol and ibuprofen is very good.

References

[1] D.J. Popowitch, E.T. Batts, C.J. Lancaster, J. Liq. Chromatogr. 1 (1978) 469.
[2] I.H. Hagestam, T.C. Pinkerton, Anal. Chem. 57 (1985) 1757.

[3] I.H. Hagestam, T.C. Pinkerton, J. Chromatogr. 368 (1986) 77.
[4] J. Haginaka, J. Wakai, Chromatographia 29 (1990) 223.
[5] H. Yoshida, I. Morita, G. Tamai, T. Masujima, T. Tsuru, N. Takai, H. Imai, Chromatographia 19 (1984) 466.
[6] C.P. Desilets, M.A. Rounds, F.E. Regnier, J. Chromatogr. 544 (1991) 25.
[7] J. Haginaka, J. Wakai, Anal. Chem. 62 (1990) 997.
[8] T. Arvidsson, K.-G. Wahlund, N. Daoud, J. Chromatogr. 317 (1984) 213.
[9] W. Roth, K. Beschke, R. Jauch, A. Zimmer, F.W. Koss, J. Chromatogr. 222 (1981) 13.
[10] G. Tarnai, H. Imai, H. Yoshida, Chromatographia 21 (1986) 519.
[11] H. Irth, R. Tocklu, K. Welten, G.J. De Jong, U.A.Th. Brinkman, R.W. Frei, J. Chromatogr. 491 (1989) 321.
[12] P.O. Edlund, D. Westerlund, J. Pharm. Biomed. Anal. 2 (1984) 315.
[13] H. Irth, R. Tocklu, K. Welten, G.J. De Jong, U.A.Th. Brinkman, R.W. Frei, J. Chromatogr. 491 (1989) 321.
[14] J. Hermansson, A. Grahn, J. Chromatogr. A 660 (1994) 119.
[15] K.-S. Boos, A. Rudolphi, S. Vielhauer, A. Wolfort, D. Lubda, F. Eisenbeiss, Fresenius J. Anal. Chem. 352 (1995) 684.
[16] J. Hermansson, G. Schill, in: P.A. Brown, R.A. Hartwick (Eds.), High-Performance Liquid Chromatography (Monographs on Analytical Chemistry Series), Wiley-Interscience, New York, 1988, pp. 337–374.
[17] N.W. Tietz (Ed.), Clinical Guide to Laboratory Tests, W.B. Saunders, 1983, pp. 586 and 606.
[18] I. Mignot, N. Presle, F. Lapicque, C. Monot, R. Dropsy, P. Netter, Chirality 8 (1996) 271.
[19] G. Schill, H. Ehrsson, J. Vessman, D. Westerlund, Separation Methods for Drugs and Realted Organic Compounds, Swedish Pharmaceutical Press, Stockholm, 2nd ed., 1983.
[20] L.B. Nilsson, D. Westerlund, Anal. Chem. 57 (1985) 1835.